Assessment of transcriptional responses of *Bacillus subtilis* cells to the antibiotic enduracidin, which interferes with cell wall synthesis, using a high-density tiling chip

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ABSTRACT

Within the soil microenvironment, bacteria compete for limited nutrients by the production of antibiotics that serve to inhibit the growth of their competitors. At the same time, bacteria have developed defense systems to cope with the external antibiotic challenges. The cell envelope is the target for numerical antibiotics, and, in Gram-positive bacteria, its alterations and dysfunctions by antibiotics are mainly sensed by two class of signal transduction systems, ECF sigma factors and two-component systems (TCSs) to induce appropriate counter actions to repair damage and secure functionality. In both systems, membrane-embedded sensors (anti-sigma factors and sensor histidine kinases) activate cognate transcriptional regulators (ECF sigma factors and response regulators) upon signal perception. Recent transcriptomic approaches to comprehensively identify genes up-regulated in the presence of various antibiotics in a model Gram-positive bacterium, *Bacillus subtilis*, have revealed the involvement of three ECF sigma factors, SigM, SigW and SigX, and five TCSs, LiaRS, BceRS, YvcPQ, YxdJK, and YhcZY.

Among these TCSs and genes controlled by them, the *lia* operon under the control of LiaRS TCS has been paid attentions, since this operon was induced by several environmental stresses and antibiotics. It consists of six genes, *liaI*, *liaH*, *liaG*, *liaF*, *liaS*, and *liaR*. Increase in the level of *liaI* and *liaH* expression has been reported by alkaline and ethanol shock condition, secretion stress due to AmyQ over expression,

and the addition of several peptide antibiotics e.g. bacitracin, nisin, ramoplanin, vancomycin, fliurimicin B, and very recently daptomycin. However, biological role of the LiaRS TCS is unclear, because its inactivation showed no phenotype, except very recent finding that *liaRS* and *liaH* deletion resulted in increased sensitivity to Daptomycin. Independent of this new report, I found that enduracidin, a peptide antibiotic interfering with transglycosylation of lipid II, induced *lia* operon strongly in very low concentration, and LiaI and LiaH directly contribute to the enduracidin resistance.

During the course of preliminary experiments to survey antibiotics that activate YvcPQ and YxdJK two component systems, we found that the YvcPQ system is activated by enduracidin. Thus, we assessed the *B. subtilis* responses to enduracidin, by using a high-density tiling oligonucleotide chip we developed. Gene arrays that have been used for previous trascriptome analyses of genes induced by antibiotics measures only signal intensity ratios compared to those from cells before the addition of antibiotics, ignoring absolute expression levels. Genes classified as “up-regulated” by this procedure may contain genes with small changes in expression levels and without biological significances. In contrast, tiling chip enabled us to monitor absolute changes in transcription levels. We analyzed transcriptome profiles at 10 and 30 min after the addition of a sub-growth-inhibitory concentration of enduracidin. Responses to bacitracin, that are most well characterized in *B. subtilis,* are also analyzed to evaluate

our analysis method.

We first extracted genes that are induced more than 5 hold or more than 10,000 units at some condition after the drug addition. Among 129 genes thus extracted, 33 genes belonged to the SigM regulon and were induced by both antibiotics. These results are consistent with the previous report suggesting that bacitracin specifically induces the SigM regulon. We also found that five TCSs described above are activated at 10 min of induction, although the induction was weak and reduced at 30 min for YvcPQ, YxdJK, and YhcZY. In contrast, expressions of *liaIH* and other member of *lia* operon were very high and maintained at 30 min. The expression of *bceAB*, ABC transporter regulated by the *bceRS* TCS, was highly induced only in the presence of bacitracin stress.

Next, we examined the effect of inactivation of TCSs on the enduracidin sensitivity, and found that *liaRS*, *liaI* and *liaH* mutants confer the sensitivity to very low concentration of enduracidin, but not *liaG* or *liaF* mutant. Additionally, we expressed *liaH* under the control of P*xyl* in the *liaH* mutant to confirm the phenotype of the *liaRS* mutant is resulted from the absence of the *liaH* expression. Furthermore we performed the Chromatin Affinity Precipitation (ChAP)-chip assay for LiaR, demonstrating that it directly regulates only the *lia* operon expression. Analysis of transcription start site and the LiaR binding region of the *lia* promoter were carried out to support this data.

In conclusion, we demonstrated that the transcriptome profiling using high-density tiling chip gives a clear view of *B. subtilis* responses to antibiotics compared to analyses

using gene arrays. We also found the direct involvement of LiaIH in the enduracidin resistance, and this finding will contribute to further understanding of molecular action of LiaIH.

CONTENT

I. INTRODUCTION

Many microorganisms living in soil produce antibiotics that inhibit the growth of competitors for limited nutrients in the environment. At the same time, microorganisms have developed defense systems to cope with external antibiotic challenge. The cell envelope is the target of many antibiotics, and, in Gram-positive bacteria, membrane alterations and dysfunction caused by antibiotics are sensed mainly by two classes of signal transduction systems: the ECF (extracytoplasmic function) sigma factors and the two-component signal transduction systems (TCSs). These systems induce appropriate counteractions to repair membrane damage and to ensure membrane functionality (Jordan et al., 2008). With both systems, membrane-embedded sensors (anti-sigma factors and sensor histidine kinases) activate cognate transcriptional regulators (ECF sigma factors and response regulators) upon signal perception. Recent transcriptomic approaches using gene arrays to comprehensively identify genes upregulated in *Bacillus subtilis* challenged with sub-inhibitory concentrations of antibiotics that interfere with cell envelope integrity, including vancomycin (Cao et al., 2002; Eiamphungporn and Helmann, 2008), bacitracin (Mascher et al., 2003), cationic antimicrobial peptides (CAMPs) (Pietiainen et al., 2005), daptomycin (Hachmann et al., 2009; Wecke et al, 2009), and friulimicin B (Wecke et al, 2009), have revealed that combinations of three ECF sigma factors (SigM, SigW, and SigX) and five TCSs (LiaRS, BceRS, YvcPQ, YxdJK, and YhcZY) are activated in response to cell envelope stress caused by antibiotic action (Figure 1).

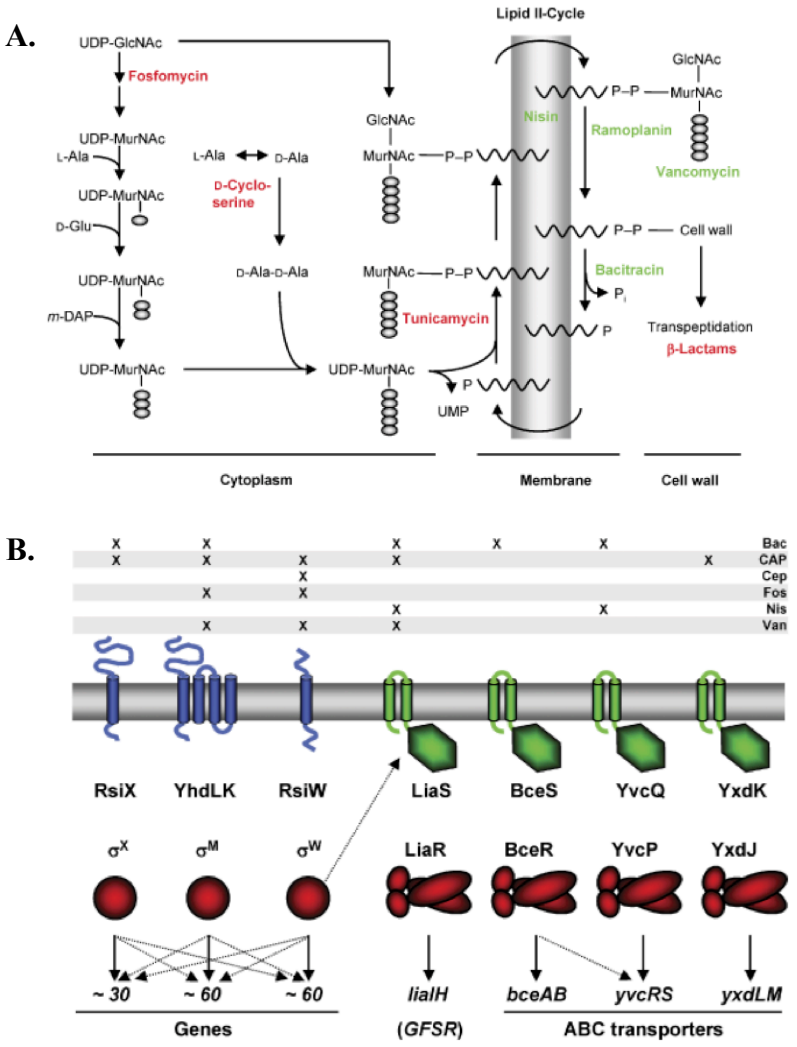

Figure 1. Cell wall biosynthesis and its inhibition by antibiotics (A) and transcriptional response of *B. subtilis* cells to antibiotics (B) (Jordan et al. 2008). (A) Important steps in cell wall biosynthesis are schematically depicted, and their cellular location is indicated below. GlcNAc, N-acetyl-glucosamine; MurNAc, N-acetyl-muramic acid. Amino acids are symbolized by small grey circles. The steps of cell envelope biosynthesis linked to the cytoplasmic membrane through undecaprenol (symbolized by the waved lines) are referred to as the 'Lipid II cycle'. This name is based on its central intermediate, Lipid II, which consists of the GlcNAc/MurNAc-pentapeptide building block, covalently linked to the lipid carrier molecule undecaprenol via a pyrophosphate ester bridge. Antibiotics in green sequester the substrate of the given step; those in red inhibit the corresponding enzymatic function. (B) The regulatory network of ECF sigma factor and TCS in *B. subtilis*. Dotted lines indicate cross-regulation. The antibiotic specificity for each system is shown above. Bac, bacitracin; CAP, CAMPs (note that individual peptides will only induce a certain subset of the regulators indicated); Cep, cephalosporin C; Fos, fosfomycin; Nis, nisin; Van, vancomycin. It should be noted that because of the known regulatory overlap between ECF σ factors, a clear assignment of the inducer spectrum, is not as unambiguously possible as indicated in this figure. In contrast, the 'crosstalk' observed for BceRS-like TCS is most likely an experimental artifact without relevance in vivo.

I.1. ECF sigma factors

The extracytoplasmic function (ECF) sigma factors are found in a diverse range of bacteria and many are activated to transcribe their regulons in response to a change in environmental conditions. The ECF sigma factors have distinct structure characteristics in region 2, which is responsible for binding core RNA polymerase and recognition of -10 region of promoter sequences, distinguishing them from other sigma factors (Raivio and Silhavy, 2001). In many cases, the ECF sigma factor is co-transcribed with one or more negative regulators. Often, these include a trans-membrane protein with an extracytoplasmic sensory domain and an intracellular inhibitory domain functioning as an anti-sigma factor that binds and inhibits the cognate sigma factor. Activation and release of ECF sigma into cytoplasm appear in sensing of environmental signal by anti-ECF sigma factor trough interaction of periplasmic domain with other envelope-localized regulatory proteins. The ECF sigma factor in cytoplasm can associate with RNA polymerase and mediate transcriptional activation of gene targets.

The *B. subtilis* genome encodes seven ECF sigma factors, and five of them have been demonstrated to be regulated by anti-sigma factors encoded in the same operon encoding cognate ECF sigma factor genes (Figure 2) (Yoshimura, et al., 2004). The physiological roles of four of these (SigM, SigW, SigX, and SigY) have been investigated in some detail. It has been shown that the proteins act on partially overlapping regulons related to cell envelope homeostasis and antibiotic resistance (reviewed in Mascher et al., 2007). The roles of the other three factors (SigV, SigZ, and YlaC) remain elusive. The direct involvement of an ECF sigma factor in mediating resistance to antibiotics has been demonstrated for SigW, which regulates resistance genes for fosfomycin, sublancin, and the antimicrobial peptide SdpC (Butcher and

Helmann, 2006). SigM induces a bacitracin-resistance determinant, *bcrC* (Cao and Helmann, 2002; Bernard et al., 2005). SigX regulates the *dltA* and *pssA* operons that affect the overall net charge of the cell envelope by incorporating positively charged groups into teichoic acids and the cytoplasmic membrane, respectively. Consistent with these observations, a *sigX* mutant strain displayed increased sensitivity to CAMPs (Cao and Helmann, 2004). However, the ECF sigma factors of *B. subtilis* recognize structurally similar promoter sequences, and regulatory overlap has been demonstrated. This often makes it difficult to evaluate the contribution of an individual system to genetic resistance to any particular antibiotic, because the effects of inactivation of one sigma factor may be compensated for by other factors. Indeed, triple inactivation of the *sigM*, *sigW*, and *sigX* genes in an undomesticated *B. subtilis* strain resulted in a greatly increased sensitivity to several cell wall-active antibiotics. In a number of instances, the antibiotic-sensitive phenotypes were significantly enhanced in the triple mutant strain relative to strains lacking only one or two factors (Mascher et al., 2007).

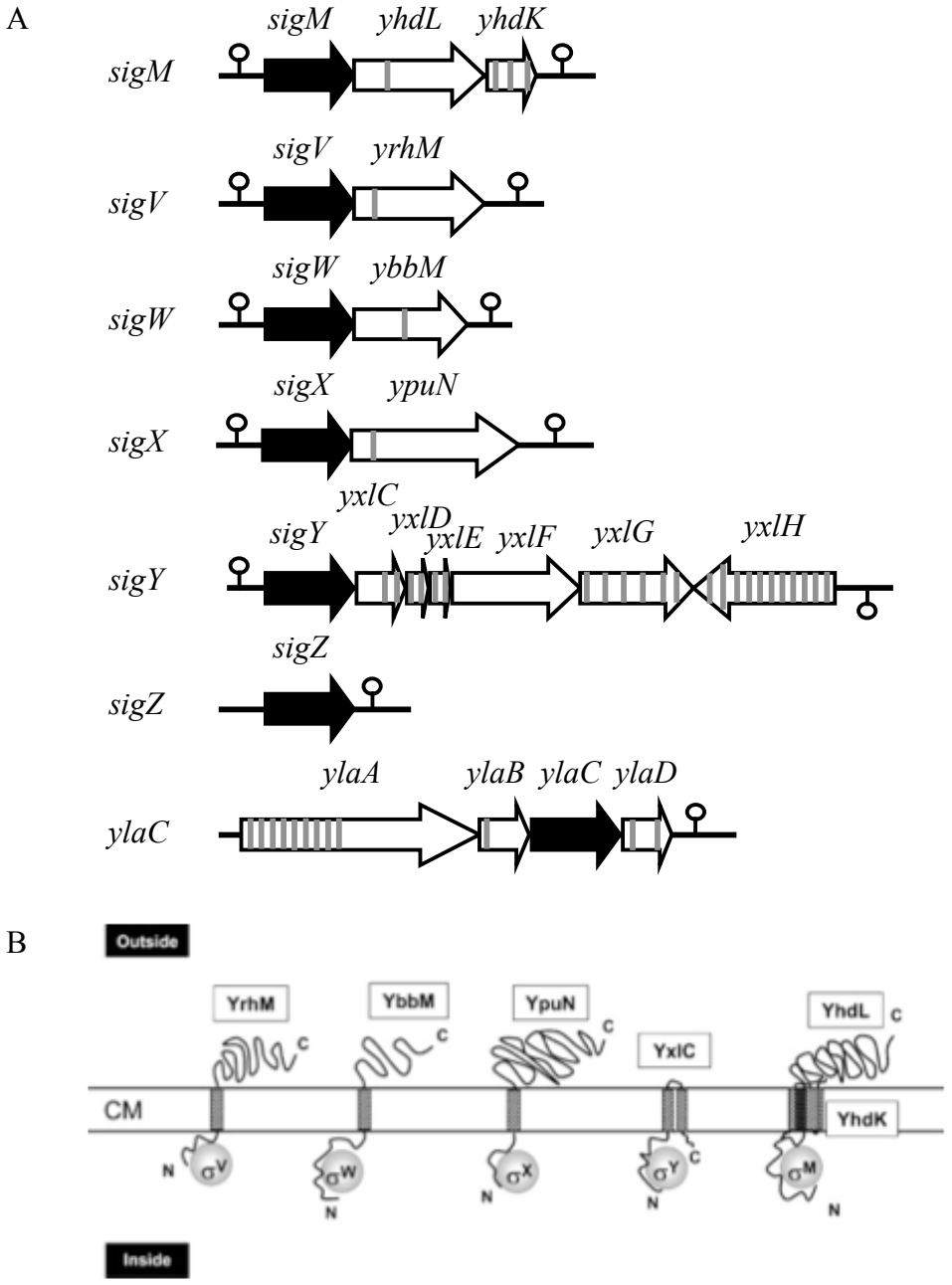

Figure 2. *B. subtilis* operons encoding *ECF* sigma factors (Yoshimura et al., 2004). (A) Gene organization of the ECF sigma factor operon in *B. subtilis*. Stem and loop structures represent putative transcription terminators. Vertical grey lines indicate probable membrane-spanning domains, predicted by the program SOSUI (http://sosui.proteome.bio.tuat.ac.jp). (B) Model of the interactions between ECF sigma factors and anti-sigma factors. Hatched boxes indicate predicted trans-membrane domains. CM, cytoplasmic membrane.

I.2. Two-component systems

The prototypical two-component systems (TCSs) are consisting of a usually membrane-bound sensor histidine protein kinase (HK) and a response regulator (RR), most often mediating differential gene expression Figure 3. The HK harbors an N-terminal input domain that senses a specific stimulus, and the information is transduced through intramolecular conformational changes, resulting in the activation of the cytoplasmic transmitter domain. The transmitter, in turn, activates its cognate receiver, encoded by the N-terminal domain of the RR. The RR gives rise to the appropriate cellular response, which is mediated by the C-terminal effector (or output) domain of the RR through protein-protein interaction (e.g., chemotaxis) or protein-DNA interactions leading to differential gene expression (Parkinson and Kofoid, 1992; Stock, et al., 2000).

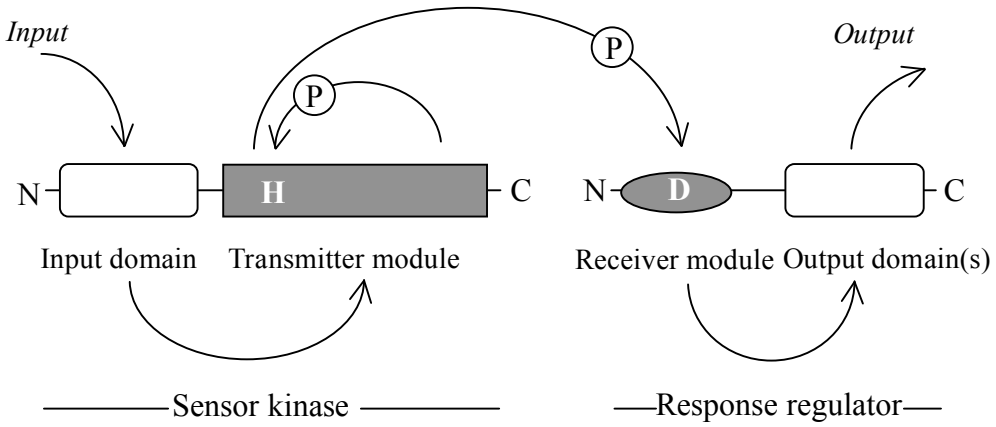

Figure 3. The "two-component" paradigm for sensory signaling via communication module (Parkinson and Kofoid, 1992). Information flows by one domain upon another (crosshatched arrows) and by phosphorylation reactions (arrow labeled P) involving histidine (H) and aspartate (D) residues.

The physiological roles of TCSs in mediating resistance to antibiotics are relatively unclear (Figure 4). BceRS induces the expression of downstream *bceAB* genes encoding an ABC transporter that facilitates bacitracin removal (Ohki et al., 2003). YvcPQ and YxdJK are structurally homologous to BceRS and induce the expression of downstream *yvcRS* and *yxdLM*, respectively, which are homologous to *bceAB* (Joseph et al., 2004; Giyanto, unpublished results). Expression of *yxdLM* and *yvcRS* is induced by CAMP LL-37 (Pietiainen et al., 2005) and nisin (Hansen et al., 2007), respectively. However, no phenotypic change has been detected in *yxdLM* or *yvcRS* mutants. The LiaRS system activates expression of the *liaIHGFSR* operon (Mascher et al., 2004). The expression of the *lia* operon is strongly induced by antibiotics that interfere with the lipid II cycle of peptidoglycan synthesis; these antibiotics include bacitracin, nisin, ramoplanin, and vancomycin (Mascher et al., 2004). However, *liaRS* inactivation did not affect the sensitivity of *B. subtilis* cells to these antibiotics. In addition, LiaRS is proposed to induce the expression of the *yhcZY-yhdA* operon, the biological role of which is unknown (Jordan et al., 2006).

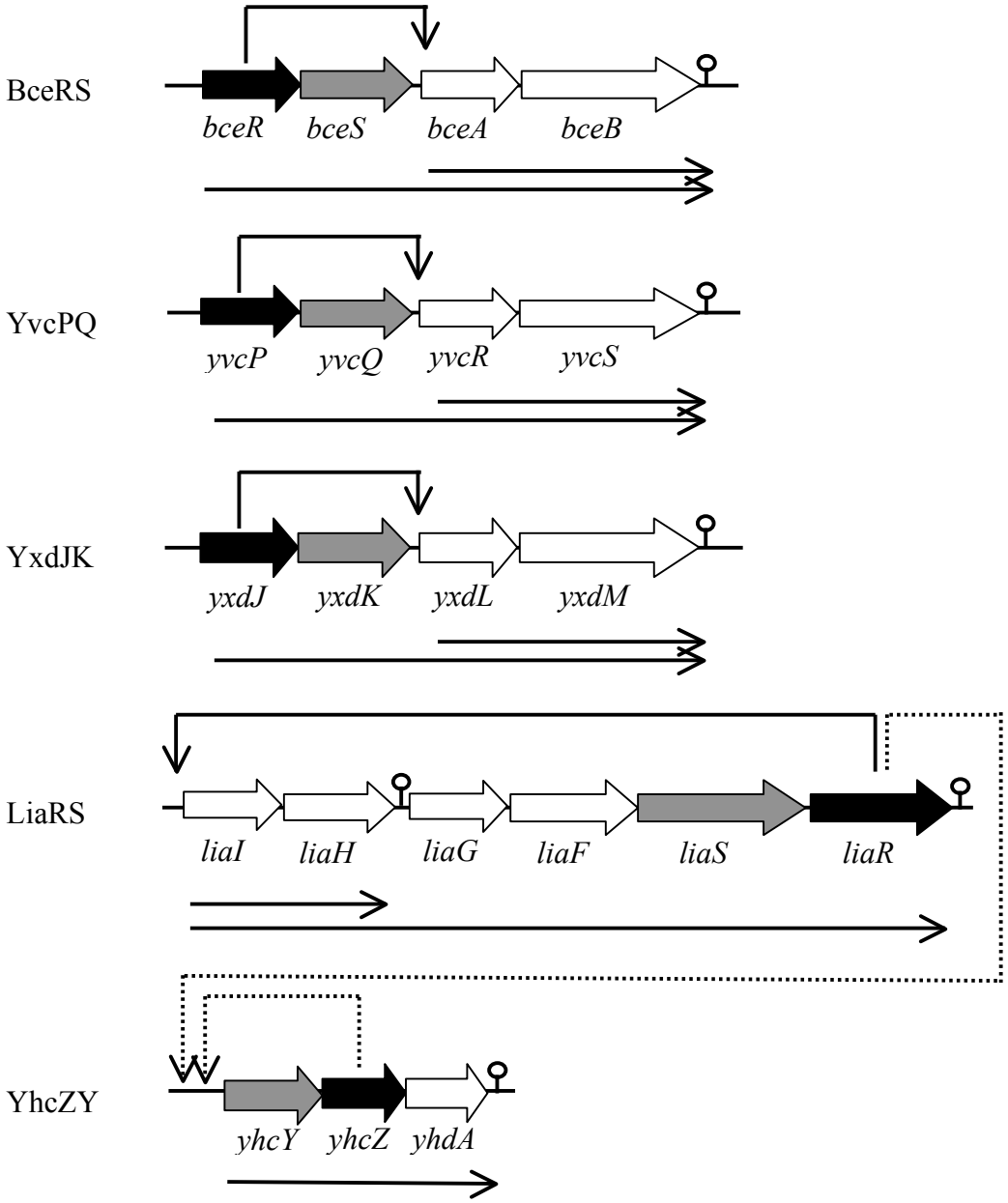

Figure 4. Operon structure of TCS genes specifically induced by cell envelope stress caused by antibiotics. Gene arrangements of operons containing TCS genes known to be specifically induced by cell envelope stress caused by antibiotics are indicated by bold arrows. Grey and black arrows show sensor kinase and response regulator genes, respectively. Transcriptional units and target sites of response regulators are indicated by arrows below and above gene maps, respectively. Dashed lines indicate putative regulatory circuits. The circle on a stem represents a transcriptional termination signal.

I.3. The *lia* operon

Notably, the *liaH* gene in *lia* operon encodes a protein homologous to the *Escherichia coli* phage-shock protein PspA, which is induced under membrane stress conditions, including bacteriophage infection (Hardie et al., 1996), extreme heat and osmotic shock (Breissette et al., 1990), exposure to organic solvents (Kobayashi et al., 1998), and prolonged incubation under alkaline conditions (Weiner and Model, 1994). The *psp* operon encodes five proteins (Figure 5); PspA, encoded by the first gene of operon, is a 26-kDa protein localized both peripherally bound to the inner membrane and in the cytosol (Kleerebezem et al. 1993; Adams et al. 2003). PspB is anchored in the inner membrane by a hydrophobic N-terminal segment and has a C-terminal cytoplasmic domain. The PspC, consist of an N-terminal cytoplasmic domain, a single trans-membrane segment, and a periplasmic C-terminal domain containing a leucine zipper motif, and PspD also localizes as peripherally bound inner membrane protein. Finally, PspE is a rhodanese-related enzyme (Adams et al. 2002). Transcription of the *pspABCDE* is initiated from a promoter located upstream of *pspA* by σ^{54}-containing RNA polymerase, and the expression requires an activator, PspF, encoded by a gene located upstream of the *psp* operon (Reitzer and Schneider, 2001; Brissete et al., 1991). PspF is constitutively active in vivo or in vitro (Javanovic et al. 1997; Javanovic et al. 1999). In uninduced condition of the *psp* operon, PspA binds to PspF by protein-protein interaction to prevent it from activating transcription. In condition which may reduce the proton motive force, then PspF is released from the PspA binding and then activates the *psp* operon promoter (Dworkin et al., 2000; Adams et al., 2003). Upon induction, PspA becomes abundant and associate with cytoplasmic membrane and serves to maintain proton motive force. In addition, mutation in the *pspA* gene resulted in the

decrease of the proton motive force (PMF), suggesting that PspA is involved in the maintenance of the PMF, which may be dissipated by proton leakage (Adams et al., 2003; Darwin, 2005). Consistent with this hypothesis, recent in vitro study demonstrated that oligomers of PspA bind to membrane phospholipids and suppress proton leakage (Kobayashi et al., 2007). It is not clear whether all the different stimuli for induction of *psp* expression in *E. coli* interfere with proton translocation, but all of them probably injure the cell membrane integrity, suggesting that PspA may play a general role in maintaining the integrity of the inner membrane (Adams et al., 2003).

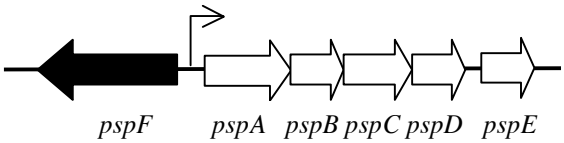

Figure 5. Structure of the *E. coli psp* operon (Darwin, 2005)

Among genes in the *psp* operon, *pspA* which plays a central role to maintain the membrane integrity in *E. coli* is conserved in wide range of bacteria. *B. subtilis* genome contain two homologous genes of *pspA*; *ydjF* regulated by SigW and *liaH* in the *lia* operon. The *B. subtilis lia* operon strongly responds to the external presence of various cell wall antibiotics. In addition, LiaRS-dependent gene expression has been reported to be induced by alkaline shock, detergents, ethanol, phenol, organic solvents, and secretion stress, albeit to a lesser extent (Jordan et al., 2006). However no phenotypic changes have been reported for *liaRS* or *liaH* inactivated *B. subtilis* cells. CAMP LL-37 (Pietiainen et al., 2005) and daptomycin (Hachmann et al., 2009; Wecke et al, 2009) are supposed to disrupt cell membrane structure and also induce *lia* operon expression, and the deletion of the *liaH* gene was very recently found to lead to a three-fold increase in

susceptibility to daptomycin (Hachmann et al., 2009). Comparative genomics analysis indicates that the *liaRS* TCS and *liaF* are conserved as operon in firmicutes, while *liaIH* is only found in group of bacilli (Figure 6).

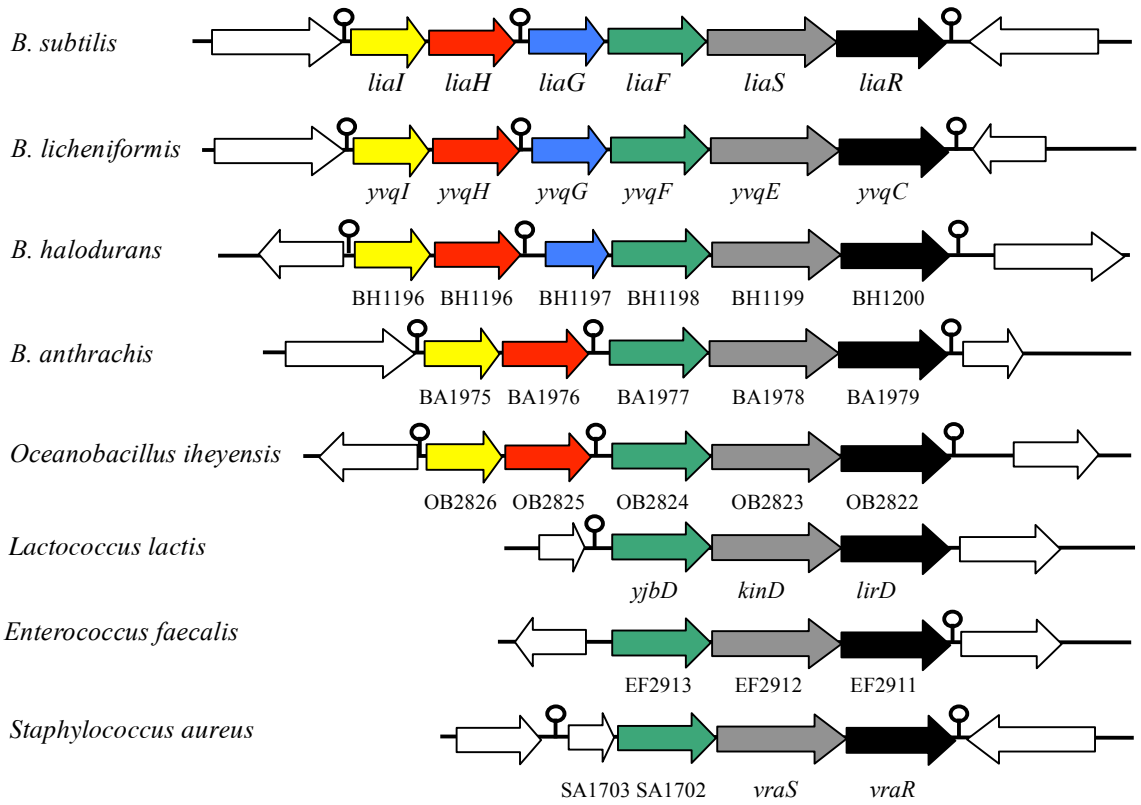

Figure 6. Comparative genomic of *lia* locus in firmicutes. Yellow, red, blue, green, grey, and black arrows indicate orthologous genes of *B. subtilis liaI, H, F, G, S, and R* genes, respectively. This picture was taken from Jordan et al. (2006) with modification.

I.4. Summary of my research

It should be noted that in addition to the ECF sigma factor and TCS regulons discussed above, many genes, the induction mechanisms and biological roles of which are difficult to evaluate, were "upregulated" in transcriptomic analyses of responses to antibiotics. Evaluation of the biological roles of such genes requires further work, although some supposed elevations may simply represent background noise caused by the accuracy limitations of gene array experiments and/or difficulties in regulating the growth conditions of target and control cells.

During the course of preliminary experiments to survey antibiotics that activate YvcPQ and YxdJK TCSs, we found that the YvcPQ system was activated by enduracidin, the target of which is considered to be the transglycosylation step of peptidoglycan biosynthesis (Fang et al., 2006). This finding prompted us to assess *B. subtilis* responses to enduracidin using a high-density tiling chip previously described by our group (Ishikawa et al., 2007; Morimoto et al., 2008). Responses to bacitracin, which are well characterized in *B. subtilis*, were also explored in order to evaluate our analysis method. We used the quantitative advantage of the tiling chip to introduce a new criterion, an increase in transcriptional level, in addition to the conventional induction ratio, to distinguish genes of biological significance from those with lower induction ratios. Our results indicate that introduction of this criterion led to unambiguous identification of core transcription responses to antibiotics, with a reduction in the number of possible background genes, compared to previous results obtained using gene arrays. We identified 129 genes that were significantly upregulated by enduracidin and/or bacitracin. Notably, we found that inactivation of LiaRS TCS, which was very strongly induced by the two antibiotics, resulted in increased sensitivity

to enduracidin, probably through a failure to induce LiaIH proteins. We noted that 33 genes belonging to the SigM regulon were induced by both antibiotics. Consistent with stronger induction of the SigM regulon in enduracidin-treated cells, inactivation of *sigM* resulted in increased sensitivity to enduracidin. In addition, and for the first time, we found that the Spx regulon was induced in cells challenged by enduracidin and bacitracin, suggesting that thiol-oxidative stress would be induced in cells challenged by antibiotics. These findings contribute to further our understanding of the molecular actions of genetic systems involved in antibiotic resistance.

II. MATERIALS AND METHODS

II.1. PCR primers and plasmids

PCR primers and plasmids used in this study are listed in Table 1 and 2, respectively.

Table 1. PCR primers used in this study

Primer	Sequence	Function
liaI-up F	5'-GGATGAATACCTTTTTGCCGC	Disruption of *liaI*
liaI-up R	5'-TCAAAAATTCCTTGTTTATTTTCATGCAGAT	Disruption of *liaI*
liaI-down F	5'-AATAAACAAGGAATTTTTGAAGAAAAAATAATATC	Disruption of *liaI*
liaI-down R	5'-CTGATTGGGTAGgatccgcgCTGTCAATTTTGTCAAATGTC	Disruption of *liaI*
liaI-mid F	5'-GAGTCAATTCCGCTGTCGGGATCTCCGTCTTTTTTGGAG	Disruption of *liaI*
liaI-mid R	5'-AGATGATACACCGTTATCAGC	Disruption of *liaI*
liaH-up F	5'-CTTGTATAAGATGAAGACA	Disruption of *liaH*
liaH-up R	5'-TTGCCGCTTTTAATACCATACCTGATTCCTC	Disruption of *liaH*
liaH-down F	5'-TATGGTATTAAAAGCGGCAAATGAATAAGC	Disruption of *liaH*
liaH-down R	5'-CTGATTGGGTAGgatccgcgCAGACACGTCACCGCTTGAGG	Disruption of *liaH*
liaH-mid F	5'-GAGTCAATTCCGCTGTCGGAAGGACTTGATAAAATGGAG	Disruption of *liaH*
liaH-mid R	5'-CGCGCGGATTTCCATTTCTTC	Disruption of *liaH*
liaG-up F	5'-GCGAAAGCTAAAGAGCATATG	Disruption of *liaG*
liaG-up R	5'-CTAGATCCCCATCCGACTCAATGACCATGCT	Disruption of *liaG*
liaG-down F	5'-TGAGTCGGATGGGGATCTAGCGATACGGTAG	Disruption of *liaG*
liaG-down R	5'-CTGATTGGGTAGgatccgcgCCAGAGACATTTAAATCGTTC	Disruption of *liaG*
liaG-mid F	5'-GAGTCAATTCCGCTGTCGGAGACTCAGGAAAGTTATTTG	Disruption of *liaG*
liaG-mid R	5'-CCTTTATCAGCAAATGAATAC	Disruption of *liaG*
liaF-up F	5'-CGCTTTCCGGGCTGAACGCAG	Disruption of *liaF*
liaF-up R	5'-CGTACTTCACCTGTTTTTTTGTCATTCCTGG	Disruption of *liaF*
liaF-down F	5'-AAAAAAACAGGTGAAGTACGTATGAGAAAA	Disruption of *liaF*
liaF-down R	5'-CTGATTGGGTAGgatccgcgGATCTCTGGCCAATCGCTGGC	Disruption of *liaF*
liaF-mid F	5'-GAGTCAATTCCGCTGTCGCCCGTGATTGGCTTGGCTCCG	Disruption of *liaF*
liaF-mid R	5'-GACGCGGCATATACCTTCGTG	Disruption of *liaF*

Table 1. (continued)

Primer	Sequence	Function
liaS-up F	5'-GGCAGAAGAAAGGAGGCG	Disruption of *liaS*
liaS-up R	5'-GGGACCTTCACTACGTACTTCACATCCACATCACC	Disruption of *liaS*
liaS-down F	5'-GTGAAGTACGTAGTGAAGGTCCCGATTTTTCCGG	Disruption of *liaS*
liaS-down R	5'-GCTGATTGGGTAGGATCCGCGGGGCTGGGAAACGAGGTC	Disruption of *liaS*
liaS-mid F	5'-CTTGAGTCAATTCCGCTGTCGCTGATCAGCGTGACCGTCG	Disruption of *liaS*
liaS-mid R	5'-CCCTTGGATATCGCTGTGTCC	Disruption of *liaS*
liaR-up F	5’-CGAGCATATGGCAGGCGAAGC	Disruption of *liaR*
*liaR*up R	5’-GATGATTTCGTACTCGAATCACGTTCGTTCT	Disruption of *liaR*
liaR-down F	5’-GATTCGAGTACGAAATCATCTCGTGAATTAG	Disruption of *liaR*
liaR-down R	5’-CTGATTGGGTAGgatccgcgCGATTGAGGTTCAAAATATCA	Disruption of *liaR*
liaR-mid F	5’-GAGTCAATTCCGCTGTCGGACGGCAGCGAAGGTGTTCGG	Disruption of *liaR*
liaR-mid R	5’-CGTCCGGTCACTGACATCCAG	Disruption of *liaR*
liaR-gst F	5’-AAGgaatccGTGATTCGAGTATTATTGATTG	*liaR* purification
liaR-gst R	5’-GAAgtcgacATTCACGAGATGATTTCGGTG	*liaR* purification
liaR-12his F	5’-GGAgtcgacCGCGCTCAGCTATCTGTTGAA	Fusion to 12his
liaR-12his R	5’-GGActcgagATTCACGAGATGATTTCGGTG	Fusion to 12his
lia Mob F	5’-CGCGTGCCAATGGGTCCGGTGCG	Mobility gel shift assay
lia Mob R	5’-CGCTCATGCAGATCCTCCTTTCG	Mobility gel shift assay
liaI probe F	5’-CAAGAAAACAATAGGCGGATTC	Northern hybridization
liaI probe R	5’-**TAATACGACTCACTATAGGGCGA**CTTCAAAAATTCTTCCCATTG	Northern hybridization
liaH probe F	5’-TGAAACAGCACACGATTGCC	Northern hybridization
liaH probe R	5’-**TAATACGACTCACTATAGGGCGA**TCGTATTCATATGCTCTTTAGC	Northern hybridization
liaG probe F	5’-GACCATCTTGCGCTTTCCGG	Northern hybridization
liaG probe R	5’-**TAATACGACTCACTATAGGGCGA**CTACCGTATCGCTAGATCCC	Northern hybridization
liaF probe F	5’-CATCACTTTAATTTATTCGGC	Northern hybridization
liaF probe R	5’-**TAATACGACTCACTATAGGGCGA**TCATCGCTTTAGATAAATCG	Northern hybridization

Table 1. (continued)

Primer	Sequence	Function
lia promt I F	5'-CGCgaatccTTGTGCCAATGGGTCCGGTGC	β-galactosidase assay
lia promt II F	5'-CGCgaatccGAGATACGACTCCGGTCTTAT	β-galactosidase assay
lia promt III F	5'-CGCgaatccATAAAAATCAATCTCTGATTC	β-galactosidase assay
lia promt IV F	5'-CGCgaatccGTTTTGCATATCTTCCAACTT	β-galactosidase assay
lia promt R	5'-CGCggatccGACGGAGATCCCAAATACAAT	β-galactosidase assay
PE1	5'-CGCGGAAGAAAGGTCCGACCTAATTAA	Primer extension
PE2	5'-CGCGGATCCCGCGGCAAACCTTTTGAT	Primer extension
lia Q F	5'-GTCCGGTGCGAGATACGAC	quantitative PCR
lia Q R	5'-TCATGCAGATCCTCCTTTCG	quantitative PCR
yhcY Q F	5'-TCAAGTATGAGCTGGGTCATTG	quantitative PCR
yhcY Q R	5'-TTTGTATTTCATTGCTTCACTTG	quantitative PCR
PF43	TGGAAATCCGAGTGAGTNNNNNNNNN	ChAP-chip
PF44	TGGAAATCCGAGTGAGT	ChAP-chip

- Small letters indicate restriction enzyme recognition sites
- Bold letters indicate the T7 promoter sequence

Table 2. Plasmid used in this study

Plasmid	Vector	Insert	Function
pDL2			Fusion to *lacZ*
pDG148			Overexpression of protein
pGEX 4T-1			Fusion to *gst*
pGEX 4T-1-*liaR*	pGEX 4T-1	Full length of *liaR*	Purification of LiaR-GST protein
pDG148*liaR*	pDG148	Full length of *liaR*	Overexpression of *liaR*

II.2. **Bacterial strains**

The *B. subtilis* and *E. coli* strains used in this study are listed in Table 3 and 4, respectively.

Table 3. *B. subtilis* strains used in this study

Strain	Genotype	Source
B. subtilis 168	*trpC2*	Lab. stock
BAB1	168 Δ*liaI*	This work
BAB2	168 Δ*liaH*	This work
BAB3	168 Δ*liaG*	This work
BAB4	168 Δ*liaF*	This work
BAB5	168 Δ*liaS*	This work
BAB6	168 Δ*liaR*	This work
BAB7	168 *liaR*-12*his*	This work
BAB8	168 *amyE*::P_{liaI}(-97 to +82) *lacZ spec*	This work
BAB8a	168 *amyE*::P_{liaI}(-78 to +82) *lacZ spec*	This work
BAB8b	168 *amyE*::P_{liaI}(-57 to +82) *lacZ spec*	This work
BAB8c	168 *amyE*::P_{liaI}(-34 to +82) *lacZ spec*	This work
BAB16	168 pDG148*liaR*	This work
sigM::*Cm*	168 *sigM*::*Cm*	Asai et al. 2008
sigV::*Cm*	168 *sigV*::*Cm*	Asai et al. 2008
sigW::*Cm*	168 *sigW*::*Cm*	Asai et al. 2008
sigX::*Cm*	168 *sigX*::*Cm*	Asai et al. 2008
sigY::*Cm*	168 *sigY*::*Cm*	Asai et al. 2008
sigZ::*Cm*	168 *sigZ*::*Cm*	Asai et al. 2008
ylaC::*Cm*	168 *ylaC*::*Cm*	Asai et al. 2008
BFS235	168 *bcrC*::pMutin	Kobayashi et al. 2003
BFS2842	168 *spx*::pMutin	Kobayashi et al. 2003

Table 4. *E.coli* strains used in this study

Strain	Genotype	Source
BL21(DE3)pLys	F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ (DE3) pLysS(cm^R)	Amersham Pharmacia Biotech
EAB1	*E. coli* BL21(DE3)pLys pGEX 4T-1-*liaR*	This work

To avoid any polar effect of selection marker fragments on transcription of downstream genes, clean deletion mutants of each gene in the *lia* operon (BAB1-6), were obtained by the method of Zhang et al. (2006), using the *mazF* gene as a counter selection marker, with a technical modification as schematically shown in Figure 7. Briefly, around 500 bp DNA fragments containing the upstream sequence and several 5'-terminal codons (fragment A), and the downstream sequence and 3'-terminal codons (fragment B) of target gene were amplified by PCR using the genome DNA of wild type cells as template, and fused by recombinant PCR using overlapping sequences introduced into appropriate primers. Around 500 bp DNA fragment containing the inner sequence of target gene was also PCR amplified (fragment C). If the gene size did not allow the amplification of internal sequence with around 500 bp in length, we amplified the sequence between ends of fragments A and B. The *E. coli mazF* gene encodes an endoribonuclease and its expression in *B. subtilis* cells is toxic. A DNA fragment containing *mazF* placed under the control of the IPTG inducible *spac* promoter (P*spac*), *lacI* controlling the P*spac* promoter, and a spectinomycin resistance (*spec*) gene was obtained by PCR using the genome DNA of the *B. subtilis* strain TMO3110 (Morimoto, unpublished) as template. Three fragments thus obtained were fused by the second recombinant PCR, and used to transform wild type *B. subtilis* cells with selection on LB plates containing 100 μg/mL spectinomycin, followed by the examination of IPTG (1

mM)-sensitive growth of transformants. Then transformants were cultivated overnight in LB liquid medium, and spread on LB agar plate containing 1 mM IPTG after an appropriate dilution, to select colonies in which the *mazF-spec* cassette was removed by intra-molecular homologous recombination.

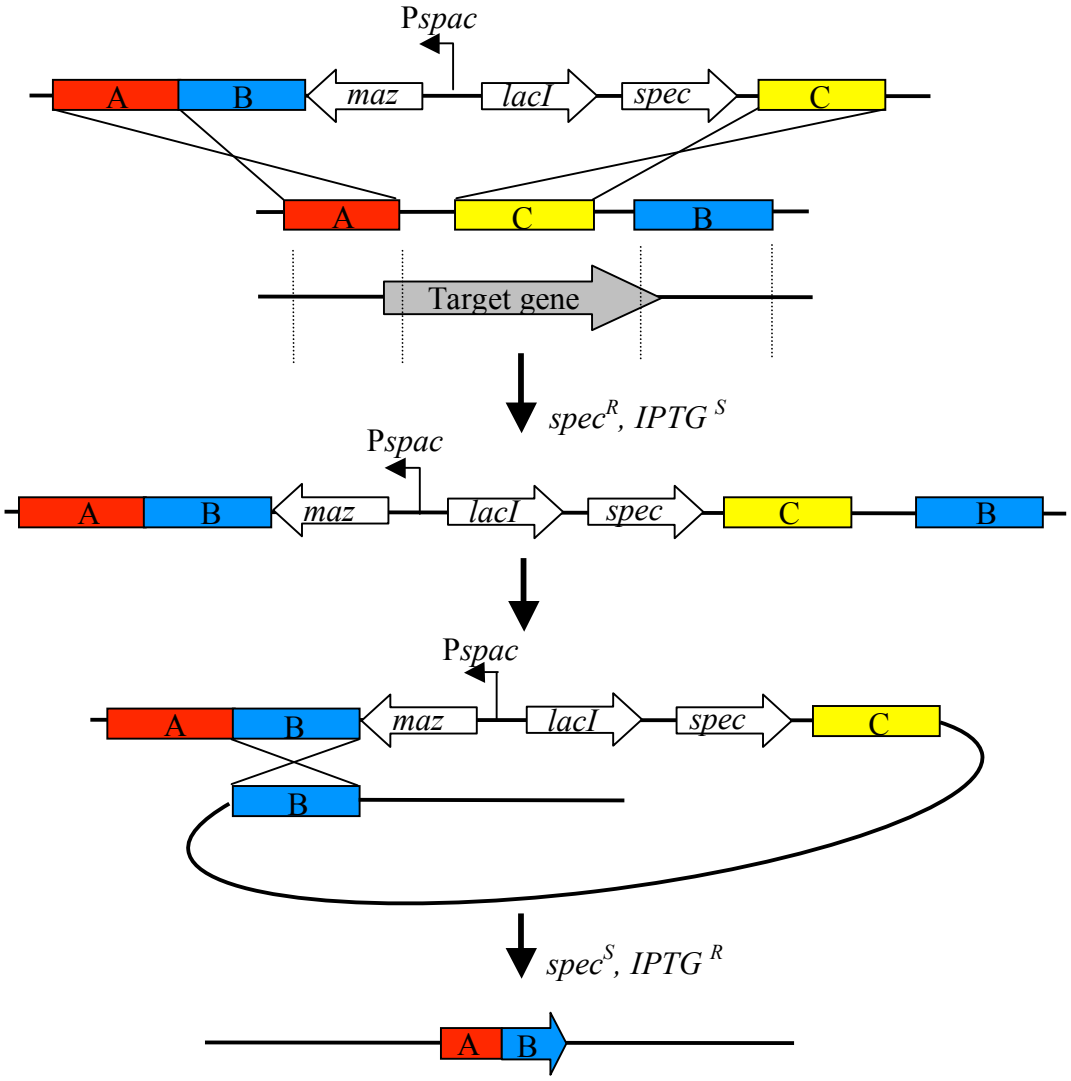

Figure 7. Clean deletion mutant construction. Construction of clean deletion mutants described in the text is schematically show.

The pMUTinHis plasmid was employed to fuse a 12x-histidine tag to the 3'-terminus of *liaR,* as described previously (Ishikawa et al., 2006), to obtain BAB7, and the pDL2 plasmid was employed to insert various fragments containing the *lia* operon promoter fused to the *lacZ* reporter gene into the *amyE* locus, as described previously (Fukuchi et al., 2000), to obtain BAB8 series strains.

To create the BAB16 strain overproducing LiaR, full length of the *liaR* gene was amplified by PCR product and cloned into the pDG148 expression vector (Kobayashi et al., 2001), followed by transfer into wild type *B. subtilis* 168 by transformation.

Disruption mutants of ECF sigma factor genes (Asai et al., 2008) were kindly provided by Dr. Kei Asai, Saitama University. Disruption mutants of *bcrC* and *spx*, constructed by an international consortium conducting *B. subtilis* genome functional analysis (Kobayashi et al., 2003), were obtained from the National BioResource Project on *Bacillus subtilis* (National Institute of Genetics, Japan).

II.3. Transcriptome analysis using a high-density tiling chip

The custom Affymetrix tiling chip used in this study contains 55,430 25-mer probes for the coding strands of protein-encoding regions, at 25-30 bp intervals, and 72,218 probes for both strands of intergenic regions, at 2-3 bp intervals (Ishikawa et al., 2007). Wild-type *B. subtilis* cells were cultivated in LB medium at 37°C to an OD_{600} value of 0.2, and divided into three portions. Cells were further cultivated for 10 or 30 min with or without 0.025 μg/mL enduracidin (MP Biomedicals) or 50 μg/mL bacitracin (Sigma).

Total RNA was extracted as described previously (Ishikawa et al., 2007). Ten mL of harvested cells by centrifugation were re-suspend in a mixture of 550 μL LETS

buffer (100 mM LiCl, 10 mM EDTA, 10 mM Tris-HCl, pH 7,4, and 1% SDS), 500 μL phenol-chloroform-isoamyl alcohol (PCIA 24:1:1) and 500 μL glass beads (0.7 mm, Asone). The mixture was then vortex at maximum speed for 4 minutes, followed by centrifugation at 8,000 rpm for 20 minutes. The supernatant was extracted again with equal volume of PCIA. The RNA from supernatant was precipitated by addition of 4.5x volume of ethanol and 1/10 volume of 4 M LiCl followed by centrifugation at 14,000 rpm. The RNA pellets were washed twice by 70% ethanol. The dried RNA pellets were suspended in 300 μL of H_2O and 900 μL 3 M sodium acetate (pH 5.6) and then incubated at -30°C for 1 hours or overnight. The RNA was collected by centrifugation, followed by washing by 70% ethanol twice, and finally re-suspend in 20 μL H_2O after dried. Purified RNA was store at -80°C until use.

Synthesis of cDNA, terminal labeling, and tiling chip hybridization were performed essentially by following the Affymetrix instruction manual, as described earlier (Morimoto et al., 2008). cDNA was synthesized from 10 μg of total RNA using random primers and reverse transcriptase (Superscript III, Invitrogen), followed by purification using Qiaquick column (Qiagen), and digested with DNase I (GE Healthcare). Using an ENZO Bioarray Terminal Labeling Kit (ENZO Life Sciences) the cDNA fragment was terminally labeled with biotin-ddUTP. Hybridization with the oligonucleotide chip was performed for 16 h at 42°C, followed by washing, staining, and scanning using the GeneChip Instrument System, according to the manufacturer's instructions (Affymetrix).

Processing of hybridization signal data was performed using the Affy-package of the Bioconductor software suite (Gentleman et al., 2004). The background of raw hybridization signals was corrected using the RMA convolution model, and

distributions of signal intensities from each experiment were normalized by the quantile method to compare signals under different test conditions (Irizarry et al., 2003). Next, based on the signal intensities of probes for each gene, individual gene transcription levels were calculated by the median polish method (Irizarry et al., 2003). The distribution of corrected transcription signals along the genome coordinate was visualized with the In Silico Molecular Cloning program, Array Edition (In Silico Biology).

II.4. Western blotting analysis of LiaR expression

Overnight culture of BAB7 cells harboring the *liaR* gene fused with a 12x-histidine coding sequence at the 3'-terminus was inoculated into 400 mL of fresh LB medium, followed by cultivation at 37°C. When the growth of cells reached to an OD_{600} value of 0.2, enduracidin or bacitracin were add to final concentration of 0.025 μg/mL and 50 μg/mL, respectively. One mL of the cells was collected by centrifugation at every 15 minutes after the addition of antibiotics. Cell pellets were dissolved in 50 μL of TE buffer containing lyzozyme (2 mg/mL) and incubated at 37°C for 10 minutes. Then 50 μL of 2x SDS loading buffer (Tris-HCl 0.1 M pH 6.8; dithiothreitol 0.2 M; SDS 4%; glycerol 20% and bromophenol blue) was added, and the mixture was incubated at 100°C for 5 minutes. The lysates were supplied to 8% polyacrylamide gel in Tris-glycine buffer (0.025 M Tris, 0.25 M glycine, 0.1% SDS), and resolved proteins were transferred on to PDVF membrane (GE Healthcare). Blots were blocked with 5% skim milk (Wako) in TBST (Tris-Buffered Saline contain 0.1% v/v of Tween 20) buffer for overnight at 4°C. Binding of rabit anti-His antibody (MBL) was performed with incubation for 1 hour with shaking at room temperature, followed by incubation with

goat anti-rabbit IgG conjugated with HRP (Biorad) for 1 hour at room temperature. The immune complexes were detected using ECL plus detection kit (GE Health care) following the instruction by the supplier.

II.5. Northern hybridization

Total RNA was extracted with the same method as decribed above. Total RNA (1 μg) was run on 1% formaldehyde-agarose gel and blotted onto a nylon membrane (Hybond N^+, Amersham Pharmachia). Hybridization was performed at 68°C overnight with digoxygenin-labeled RNA probes in hybridization buffer [5x SSC, 50 mL formamide, 0.05 M NaH_2PO_4 buffer, 20 mL 1x blocking stock solution (Roche), 1% Sodium Lauryl Sarcocinate, 7% SDS]. Templates for preparation of probes were obtained by PCR reaction with the primers possessing the T7 RNA polymerase promoter sequence, and the probes were synthesized in vitro by using T7 RNA polymerase (Roche). Membrane washing with 2x washing solution (2x SSC, 0.1% SDS) and 0.1x washing solution (0.1x SSC, 0.1% SDS) was done two times for 15 minutes of each. The non-specific region on membrane was then blocked with Blocking Reagent (Roche) in buffer I (maleic acid 0.1 M, NaCl 0.15 M). The hybridization signals were detected by adding anti-Digoxigenin-AP conjugate FAB fragment (Roche) and then exposed the membrane to X-ray film after addition of $CSPD^R$ ready to use (Roche).

II.6. Primer extension analysis

Total RNA was extracted from BAB16 cells over-producing LiaR from the pDG148-*liaR* plasmid at exponential growth (OD_{600}0.4). Then, 12 μg RNA was

incubated with 300.000 cpm end-labeled [γ^{32}P]-ATP PE2 primer in 20 μl TE containing 0.1 M KCl at 65°C for 30 minutes then 1.5 hours at room temperature. Reverse transcription was carried out at 42°C for 1 hour using a SUPERSCRIPT reverse transcriptase enzyme according to the manufacturer's instruction (GIBCO BRL). The cDNA were then electrophoresed in 6% polyacrylamide urea gel and radioactive bands were detected with imaging plate and BAS2500 scanner (Fuji Photo Film). DNA ladders as marker were created with the same end-labeled primer and a cycle sequencing kit (Takara). The template DNA for sequencing reaction was amplified using PE1 and PE2 primers.

II.7. β-galactosidase assay

BAB8 series strains, harboring various length of fragments containing the *lia* operon promoter fused to the *lacZ* reporter gene in the *amyE* locus, were grown in LB medium starting at an OD_{600} value of 0.01. When the growth of cells reached to an OD_{600} value of 0.2, enduracidin was add to final concentration of 0.025 μg/mL, followed by incubation for 30 minutes. The expression of protein was stopped by addition 50 μL of chloramphenicol (10 mg/ml) to 5 ml of culture followed by incubation on ice for 30 minutes. Bacterial protein was determined based on OD_{600} measurement. 100 μL of culture sample was mixed with 400 μl Z buffer (0.06 M Na_2HPO4, 0.04 M NaH_2PO4, 0.001 M $MgSO_4$, 0.01 M KCl, and 0.05 M β-merchaptoethanol), followed by addition of 25 μL of 0.1% SDS and 25 μL of chloroform. Clearted suspension was incubated at 28°C for 5 minutes, followed by addition of 100 μL of ONPG (ortho-Nitophenyl-β-galactoside, 4 mg/mL in 0.1 N phosphate buffer pH 7.0). The reaction was stop when yellow color appeared by

addition 250 μL of 1 M Na_2CO_3. Suspension then was centrifuged for 1 minutes at 10,000 rpm, then supernatant was subjected for measurement at OD_{420} and OD_{550}. β-galactosidase activity was calculated based on formula,

units = 1000 x [(OD420-1.75) x OD550]/t (min)/v (ml)/OD_{600}

II.8. Purification of LiaR-GST

Full length of *liaR* PCR product without stop codon was digested by *Eco*RI and *Sal*I restriction enzymes using their recognition sequences introduced in appropriate primers. The fragment was then ligated to pGEX 4T-1 (Amersham Pharmacia Biotech) digested by *Eco*RI and *Sal*I and transformed into *E. coli* DH5 to obtain the pGEX 4T-1-*liaR* plasmid. The plasmid then was transformed into *E. coli* BL21(DE3)pLys (Amersham Pharmacia Biotech), to obtain EAB1.

EAB1 cells were cultivated in LB medium overnight at 30°C, and inoculated into 800 ml of LB medium containing 100 μg/mL ampicilin to give an OD_{600} value of 0.01, followed by cultivation at 30°C. When the growth of cells reach to an OD_{600} value of 0.5, IPTG was added to give a final concentration of 1 mM, followed by incubation for 3 hours. Cells were collected by centrifugation and washed with 200 mM Tris-HCl pH 8.0. Cells were sonicated in 20 mL phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) with total process time 4 minutes (pulse on 10 seconds, pulse off 30 seconds), using ultrasonic processor machine (Astrason). Cellular debris was removed by centrifugation at 4°C, 8,000 rpm for 20 minutes. The supernatant fraction was mix with Glutathione Sepharose beads (Pharmacia Biotech). LiaR-GST was eluted using 1 mL of glutathione elution buffer (10 mM glutathione in 50 mM Tris-HCl pH 8.0). To check the isolation product, the elute then were run into 8%

SDS-polyacrylamide gel in Tris-glycine buffer (0.025 M Tris, 0.25 M glycine, 0.1% SDS) followed by Coomasie Blue staining.

II.9. Mobility gel shift assay

A 121 bp fragment encompassing –95 to +25 bp relative to start site of *liaI* was amplified by PCR. The PCR product was end-labeled by [γ^{32}P]-ATP using T4 polynucleotide kinase. Labeled DNA was separated from unlabelled DNA by Nick column (Amersham Pharmacia Biotech). Binding reaction was performed in 15 μL reaction mixture containing various amount of GST-YvqC protein (0-50 pmol) and 24.25 pmol (5000 cpm) labeled DNA in 5x binding buffer (100 mM Tris-Cl pH 7.4, 25 mM $MgCl_2$, 250 mM KCl, 250 μg/ml BSA, 10 μg/ml salmon sperm, 50% glycerol, 1 mM EDTA) containing 1 μl Poly dI-dC. The reaction mix was incubated at room temperature for 15 minutes and run in 5% poly acrylamide gel electrophoresis in 0.5x TBE buffer. Radioactive bands were detected with imaging plates and a BAS500 scanner (Fuji Photo Film).

II.10. Genome-wide determination of LiaR binding site(s)

ChAP-chip analysis (chromatin affinity purification coupled with gene-chip analysis) was performed as described previously (Ishikawa et al., 2007), using BAB7 cells harboring the *liaR* gene fused with a 12x-histidine coding sequence at the 3'-terminus. The BAB7 strain was cultured in 400 mL LB medium at 37°C. Enduracidin was added to final concentration of 0.025 μg/mL when growth of the cells reach to an OD_{600} value to 0.2, and cultivation was continued for 30 minutes. Crosslinking of protein to DNA was performed by formaldehyde method. The culture were transferred

in to 500 μL tube, and formaldehyde was added to a final concentration of 1%, followed by incubation incubated in shaker at 37°C for 30 minutes. The formaldehyde treated cells were harvested by centrifugation at 8,000 rpm for 5 minutes and washed by 1x TBS buffer (0.15 M NaCl, 0.02 M Tris-HCl pH 7.5) and stored at -80°C for at least 6 hours. The cells were suspended in 4 ml UT buffer (0.1 M HEPES, 0.05 M imidazole, 0.5 M NaCl, 8 M urea, 1% Triton-X, 0.01 M -merchapto ethanol, and 0.001 M PMSF), and then disrupted by sonication with total process time 10 minutes (Level 5, pulse on 10 seconds, pulse off 4 seconds) on ice, and then centrifuged at 8,000 rpm for 10 minutes. The supernatant was transferred into new tube and 50 μL of DynaTALONS beads were added, followed by incubation on shaker at room temperature for overnight. The LiaR-12His-DNA complex was separated from non-target protein-DNA complex, and washed with UT buffer. Purified LiaR-12His-DNA complex was eluted with elution buffer (0.1 M Tris-HCl pH 7.5, 0.5 M Imidazole pH 7.5, 1% SDS, and 0.01 M DTT). The eluate was washed with M-wash buffer (0.1 M Tris-HCl pH 7.5, 1% SDS, and 0.01 M DTT) and concentrated by using Microcon YM 100 (Milipore) to 50 μL. To isolate the DNA fragment bound by LiaR, the crosslinking was performed at 65°C overnight, followed by purification using Qiagen PCR clean up kit (Qiagen).

Amplification of purified fragment DNA was done using PF43 and PF44 primers (Herring et al. 2005). The PCR reaction was performed for 20 cycles using KOD-plus-DNA polymerase (Toyobo), followed by digestion with DNaseI (FPLCpure^{TM}). DNA fragments were terminally labelled and hybridized with the oligonucleotide chip. Signal intensities from mismatch probe were subtracted from perfect match probes. The signal intensities of DNA in affinity-purified fraction and DNA isolated from the whole cell extract fraction before purification were adjusted to

confer a signal average 500. Signal intensities of DNA in affinity-purified fractions were subtracted by control DNA to obtain protein-binding signals. The distribution of protein-DNA binding signals along the genome were visualized with the Molecular Cloning program, Array Edition (*In Silico* Biology),

For quantitative PCR analysis, DNA fragments that co-purified with LiaR and those in the supernatant fraction used for affinity purification were extracted from the same amounts of cells, as in ChAP-chip analysis. Purified DNA fragments were dissolved in 50 μL H_2O, followed by two-fold serial dilution, and used as templates for quantitative PCR. Promoter sequences of the *lia* and *yhcZY-yhdA* operons were amplified using KOD-plus-DNA polymerase (Toyobo) in the presence of 1.5 mM $MgSO_4$, employing *yhcY* F-*yhcY* R and *lia* F-*lia* R primer pairs, respectively. PCR was performed for 30 cycles using 1 μL aliquots of diluted templates in 10 μL volumes of reaction mixture, and 6 μL aliquots of products were separated in 2% (w/v) agarose gels, followed by ethidium bromide staining.

II.11. Spot assay to determine sensitivity of *B. subtilis* to enduracidin and bacitracin

Overnight cultures of wild-type and mutant cells, in LB medium at 37°C, were diluted to an OD_{600} value of 1.0, and further serially diluted 10-fold in LB medium. Next, 2 μL aliquots of cell suspensions were spotted onto LB agar plates with and without antibiotics, and incubated overnight at 37°C.

III. RESULTS AND DISCUSSION

III.1. Transcriptome analyses of *B. subtilis* cells under enduracidin and bacitracin stress, using the high-density tiling chip

Enduracidin is a lipodepsipeptide produced by the soil bacterium *Streptomyces fungicidicus* (Higashide et al., 1968). The antibiotic exhibits strong bactericidal activity against a broad spectrum of Gram-positive bacteria, including strains resistant to known antibiotics, and is thus an attractive target for further antibiotic development studies (Yin and Zabriskie, 2006). The primary mechanism of action is considered to be inhibition of the transglycosylation step of peptidoglycan biosynthesis, mediated by antibiotic-binding to the lipid II substrate (Fang et al., 2006). Bacitracin is a cyclic dodecylpeptide synthesized by *Bacillus licheniformis* and some strains of *B. subtilis*. The antibiotic binds to undecaprenyl pyrophosphate (UPP) and prevents the dephosphorylation of UPP necessary for recycling of the lipid carrier (reviewed in Rietkotter et al., 2008).

We analyzed changes in transcriptome profiles after addition of 0.025 μg/mL enduracidin or 50 μg/mL bacitracin, which are sub-inhibitory for *B. subtilis* growth in LB liquid medium, using a custom Affymetrix tiling chip developed earlier by our group (Ishikawa et al., 2007). Western blot analysis using BAB7 cells expressing LiaR fused with a 12x-histidine tag and antibody against the His-tag indicated that expression of the *lia* operon was maximally induced after 30 min of enduracidin addition (Figure 8), whereas northern blot analysis indicated that the expression of the *yvcRS* genes peaked at 10 min (Giyanto, unpublished). Therefore, we analyzed transcriptome profiles at 10 and 30 min after addition of antibiotics, in comparison with the profiles of cells

incubated without any drug. Responses to bacitracin, which are well characterized in *B. subtilis*, were also determined to evaluate our new analysis method using the tiling chip. After background correction of raw transcriptional signal intensities from each experiment, and normalization of the intensity distribution of each experiment to permit comparison of signals obtained under different test conditions, we calculated transcription levels (as units) of each gene under all experimental conditions explored, yielding a maximum expression value of about 45,000 units. Graphical representations of transcriptome profiles thus obtained are available in Supplementary Figure 1.

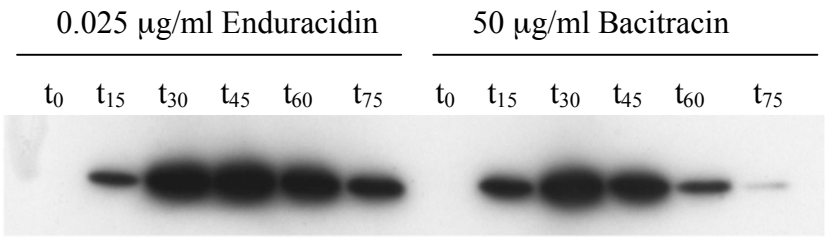

Figure 8. Time course of LiaR expression. Western blotting analysis by using BAB7 strain which was grown at 37˚C. Antibiotic addition were done at OD_{600} value = 0.2. t_0 –t_{75} indicated time of sampling point, where t_0 was sampling point just before antibiotic addition.

Next, using the quantitative advantage offered by tiling chip data, we searched for genes that were significantly upregulated in cells challenged by antibiotics, using two criteria. First, the transcription level was required to be increased by more than five-fold compared to that of control cells without drug addition (the induction ratio test). This is a common but stringent criterion in transcriptome analysis. Second, the difference in transcription levels between antibiotic-challenged cells and control cells (the induction level test) had to be significant, to permit identification of genes of biological

significance among genes with lower induction ratios, assuming that a high level of induction would correlate with biological activity. We set the minimum value of the second criterion as 10,000 units, to extract a number of genes similar to that identified by the first criterion. These procedures identified 21-53 genes under each experimental condition; these genes are listed in Table 5.

Table 5. Genes significantly up-regulated by enduracidin and/or bacitracin

		10 min							30 min						
		Signal Intensity			+End		+Bac		Signal Intensity			+End		+Bac	
Gene	Product	Cont	+End	+Bac	Ratio	Dif	Ratio	Dif	Cont	+End	+Bac	Ratio	Dif	Ratio	Dif
(Two component ststem regulon)															
liaI	conserved membrane protein	944	43568	43600	**46.17**	**42624**	**46.20**	**42657**	1863	44832	42878	**24.06**	**42969**	**23.02**	**41015**
liaH	similar to phage shock protein	1012	39325	39843	**38.84**	**38312**	**39.36**	**38831**	2110	42322	40236	**20.06**	**40213**	**19.07**	**38127**
liaG	conserved protein	2758	32339	35281	**11.73**	**29581**	**12.79**	**32523**	2103	32287	26050	**15.36**	**30184**	**12.39**	**23947**
liaF	conserved protein	2494	28158	27824	**11.29**	**25664**	**11.16**	**25330**	2337	26066	20897	**11.16**	**23730**	**8.94**	**18561**
liaS	two-component sensor histidine kinase.	2404	29874	30517	**12.42**	**27469**	**12.69**	**28113**	2598	24649	19144	**9.49**	**22051**	**7.37**	**16546**
liaR	two-component response regulator	1794	26078	26122	**14.53**	**24284**	**14.56**	**24328**	1990	15574	10722	**7.83**	**13584**	**5.39**	8732
bceR	two-component response regulator	934	4953	1316	**5.30**	4019	1.41	382	1283	2052	1685	1.60	769	1.31	402
bceS	two-component sensor histidine kinase	782	3991	1125	**5.10**	3210	1.44	343	1124	1812	1564	1.61	688	1.39	441
bceA	bacitracine efflux ABC transport system ATP-binding protein	295	2342	27430	**7.94**	2047	**93.02**	**27135**	493	830	29986	1.69	338	**60.88**	**29494**
bceB	bacitracine efflux ABC transport system permease	440	3011	29224	**6.85**	2571	**66.44**	**28784**	610	1210	31756	1.98	600	**52.08**	**31146**
yvcP	t wo-component response regulator	2019	2431	2207	1.20	412	1.09	188	2574	1976	2319	0.77	-598	0.90	-255
yvcQ	two-component sensor histidine kinase.	1732	2353	2082	1.36	621	1.20	350	2521	2062	2410	0.82	-458	0.96	-111
yvcR	ABC transport system ATP-binding protein	2272	35277	13588	**15.53**	**33004**	**5.98**	**11316**	3270	6753	6739	2.06	3482	2.06	3469
yvcS	ABC transport system permease protein	1702	30625	12335	**17.99**	**28923**	**7.25**	**10633**	2317	6008	5178	2.59	3691	2.23	2861
yxdJ	two-component response regulator	1363	1772	2041	1.30	409	1.50	679	1744	1485	1500	0.85	-259	0.86	-243
yxdK	two-component sensor histidine kinase.	1112	1160	1465	1.04	47	1.32	353	1305	1092	1137	0.84	-213	0.87	-168
yxdL	ABC transport system ATP-binding protein	327	5336	1107	**16.30**	5009	3.38	779	455	537	752	1.18	82	1.65	297
yxdM	ABC transport system permease protein	320	4509	936	**14.09**	4189	2.93	616	454	476	589	1.05	22	1.30	135
yxeA	conserved protein	457	4620	1172	**10.12**	4164	2.57	715	696	850	1125	1.22	154	1.62	429
yhcY	two-component sensor histidine kinase	910	6860	8064	**7.54**	5950	**8.86**	7154	1178	4509	1833	3.83	3331	1.56	655
yhcZ	two-component response regulator	958	7847	9199	**8.19**	6888	**9.60**	8241	1252	6273	2173	**5.01**	5021	1.74	921
yhdA	NADPH:FMN oxidoreductase	622	4637	5515	**7.46**	4016	**8.87**	4893	1064	4785	1646	4.50	3721	1.55	583
(CssRS regulon)															
htrA	serine protease	1497	10982	7476	**7.34**	9485	4.99	5979	1898	6958	4506	3.67	5061	2.37	2608
htrB	serine protease	2218	12098	8436	**5.45**	9880	3.80	6219	2633	8999	5172	3.42	6366	1.96	2539

Table 5. (continued)

		10 min							30 min						
		Signal Intensity			+End		+Bac		Signal Intensity			+End		+Bac	
Gene	Product	Cont	+End	+Bac	Ratio	Dif	Ratio	Dif	Cont	+End	+Bac	Ratio	Dif	Ratio	Dif
(SigM regulon)															
sigM	ECF-type sigma factor	4286	16957	16028	3.96	**12672**	3.74	**11743**	3323	18432	12318	**5.55**	**15109**	3.71	8995
yhdL	anti-sigM protein	5413	16442	16379	3.04	**11028**	3.03	**10966**	4898	21687	15570	4.43	**16789**	3.18	**10671**
yhdK	function unknown and unique	584	3360	3104	**5.75**	2776	**5.31**	2520	887	7892	4872	**8.90**	7005	**5.49**	3985
abh	transcriptional regulator of transition state genes	15863	22627	22821	1.43	6765	1.44	6958	17347	30615	26236	1.76	**13268**	1.51	8890
bcrC	undecaprenyl pyrophosphate phosphatase	10342	22543	21645	2.18	**12201**	2.09	**11304**	7618	22331	17536	2.93	**14713**	2.30	9919
bscR	repressor of the bscR-CYP102A3 operon	1404	11589	11588	**8.25**	**10185**	**8.25**	**10183**	1237	10709	5669	**8.66**	9472	4.58	4432
*cyp*102A3	cytochrome P450 / NADPH-ferrihemoprotein reductase	2535	12775	13023	**5.04**	**10240**	**5.14**	**10488**	2356	13970	8732	**5.93**	**11614**	3.71	6375
divIC	cell-division initiation protein	13880	21863	23011	1.58	7983	1.66	9130	12220	22483	18090	1.84	**10264**	1.48	5871
murB	UDP-N-acetylenolpyruvoylglucosamine reductase	11262	19423	19423	1.72	8161	1.72	8161	8196	25842	22831	3.15	**17646**	2.79	**14634**
divIB	cell-division initiation protein	7962	14172	15165	1.78	6210	1.90	7202	5400	15510	13490	2.87	**10110**	2.50	8089
radC	similar to DNA repair protein	8653	18813	18031	2.17	**10160**	2.08	9378	6867	16227	15258	2.36	9360	2.22	8391
sms	DNA repair protein	3251	12136	11197	3.73	8885	3.44	7946	3340	14532	10296	4.35	**11192**	3.08	6956
disA	DNA integrity scanning protein	6778	21217	19802	3.13	**14439**	2.92	**13023**	6301	24887	17265	3.95	**18585**	2.74	**10964**
yacL	conserved membrane protein	5975	14803	14848	2.48	8828	2.48	8872	5446	19023	12634	3.49	**13577**	2.32	7188
ispD	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase	6583	15522	15266	2.36	8939	2.32	8683	6072	17925	12435	2.95	**11853**	2.05	6363
yceC	similar to tellurium resistance protein	16875	25089	23919	1.49	8215	1.42	7044	17990	38582	29159	2.14	**20592**	1.62	**11169**
yceD	similar to tellurium resistance protein	15277	22281	21800	1.46	7004	1.43	6523	16194	32808	26586	2.03	**16614**	1.64	**10393**
yceE	similar to tellurium resistance protein	13022	18676	18771	1.43	5654	1.44	5749	14036	27549	20493	1.96	**13513**	1.46	6457
yceF	similar to tellurium resistance protein	16157	20668	20607	1.28	4511	1.28	4450	16966	28070	20827	1.65	**11105**	1.23	3861
yceG	conserved protein	5107	9019	8883	1.77	3911	1.74	3775	5003	15760	9274	3.15	**10757**	1.85	4271
yceH	similar to toxic anion resistance protein	6370	10134	10220	1.59	3763	1.60	3850	6447	17281	11481	2.68	**10833**	1.78	5034
ydaH	conserved membrane protein	3133	11660	11373	3.72	8526	3.63	8240	1628	12027	8141	**7.39**	**10399**	**5.00**	6513
ydbO	probable cation efflux protein	8746	16056	16065	1.84	7310	1.84	7319	7959	19108	15593	2.40	**11150**	1.96	7635
yebC	function unknown and unique	3855	14440	14161	3.75	**10584**	3.67	**10306**	2355	20130	11901	**8.55**	**17776**	**5.05**	9546
ypbG	conserved protein	1435	6638	7011	4.62	5202	4.88	5576	1440	16611	11665	**11.54**	**15172**	**8.10**	**10225**
ypbH	regulation of ClpC protease	4935	10753	10310	2.18	5817	2.09	5374	4546	19362	14548	4.26	**14816**	3.20	**10002**
ypuA	conserved protein	14258	25958	26141	1.82	**11699**	1.83	**11882**	14086	35312	32030	2.51	**21227**	2.27	**17944**
ypuD	function unknown and unique	559	4805	4284	**8.60**	4246	**7.67**	3725	558	7361	3577	**13.19**	6804	**6.41**	3019
yqjL	involved in paraquat resistance	2801	12582	12549	4.49	9781	4.48	9748	2671	21143	15439	**7.92**	**18473**	**5.78**	**12768**
yrhH	similar to methyltransferase	921	12643	12761	**13.73**	**11722**	**13.85**	**11840**	1067	18539	11109	**17.38**	**17472**	**10.41**	**10042**
ywaC	GTP-pyrophosphokinase	1642	10150	10873	**6.18**	8508	**6.62**	9231	1612	21231	13134	**13.17**	**19619**	**8.15**	**11522**
yjbC	conserved protein	6536	19706	19706	3.02	**13171**	3.02	**13171**	6325	30186	23812	4.77	**23861**	3.76	**17487**
spx	transcriptional regulator of the oxidative stress response	10944	24512	23959	2.24	**13568**	2.19	**13015**	12971	38573	29621	2.97	**25602**	2.28	**16650**

Table 5. (continued)

		10 min							30 min						
		Signal Intensity			+End		+Bac		Signal Intensity			+End		+Bac	
Gene	Product	Cont	+End	+Bac	Ratio	Dif	Ratio	Dif	Cont	+End	+Bac	Ratio	Dif	Ratio	Dif
(SigV regulon)															
sigV	ECF-type sigma factor	436	1637	1605	3.75	1200	3.68	1168	353	3680	1881	**10.44**	3327	**5.34**	1528
yrhM	similar to anti-sigma factor	567	2021	2021	3.56	1454	3.56	1454	523	4857	2765	**9.30**	4335	**5.29**	2242
(SigX regulon)															
ywnJ	function unknown and unique	657	2430	2602	3.70	1773	3.96	1944	644	3269	1909	**5.07**	2624	2.96	1264
(Spx regulon)															
gabD	succinate-semialdehyde dehydrogenase, gamma-aminobutyrate utilization	3205	7003	6832	2.18	3797	2.13	3626	3900	18678	11459	4.79	**14778**	2.94	7559
glpK	glycerol kinase	21285	18997	21225	0.89	-2288	1.00	-61	9667	20341	11604	2.10	**10674**	1.20	1937
glpD	glycerol-3-phosphate dehydrogenase	15663	17452	17343	1.11	1789	1.11	1680	1191	9119	3766	**7.65**	7927	3.16	2574
pgcA	alpha-Phosphoglucomutase	10741	13094	13094	1.22	2353	1.22	2353	9580	19617	16662	2.05	**10037**	1.74	7082
nfrA	NADPH-linked nitro/flavin reductase	3349	7309	7019	2.18	3961	2.10	3670	4031	15718	10096	3.90	**11687**	2.50	6065
sodA	superoxide dismutase	23479	23835	23892	1.02	355	1.02	413	26712	38824	35522	1.45	**12112**	1.33	8810
trxA	thioredoxin	13591	16655	17226	1.23	3064	1.27	3636	12302	25218	21554	2.05	**12915**	1.75	9252
trxB	thioredoxin reductase	12035	16181	16181	1.34	4146	1.34	4146	9582	24469	17179	2.55	**14887**	1.79	7597
yfjR	similar to 3-hydroxyisobutyrate dehydrogenase	1066	2968	2715	2.78	1902	2.55	1649	1147	8617	4314	**7.52**	7470	3.76	3168
yhfJ	similar to lipoate-protein ligase	8646	12158	12231	1.41	3511	1.41	3585	9991	22556	19897	2.26	**12565**	1.99	9906
yojF	conserved protein	7085	11689	11345	1.65	4604	1.60	4260	6951	17324	13880	2.49	**10373**	2.00	6930
yojG	conserved protein	8061	11756	11575	1.46	3695	1.44	3514	7079	19473	15051	2.75	**12394**	2.13	7971
yqjM	xenobiotic reductase, induced by oxidative stress	1386	2135	2340	1.54	749	1.69	954	1697	9660	7129	**5.69**	7963	4.20	5432
yraA	probable intracellular protease	6501	10814	10370	1.66	4312	1.60	3869	6002	17237	13338	2.87	**11236**	2.22	7336
yrzF	probable serine/threonine-protein kinase	1299	4125	4117	3.18	2826	3.17	2818	1309	8400	4528	**6.42**	7091	3.46	3219
yrzG	Fused to yrzF	959	3585	2990	3.74	2625	3.12	2031	983	7677	4007	**7.81**	6694	4.08	3024
ytkL	conserved protein	4748	8853	8525	1.86	4105	1.80	3777	5926	17781	14305	3.00	**11856**	2.41	8379
yuaE	f unction unknown and unique	1186	3246	3246	2.74	2060	2.74	2060	1548	8287	5382	**5.35**	6739	3.48	3835
yugJ	Fe-dependent alcohol dehydrogenase family	7496	10526	10236	1.40	3031	1.37	2740	7334	23813	17009	3.25	**16479**	2.32	9674
yugP	Predicted Zn-dependent protease	1132	3296	3136	2.91	2165	2.77	2004	1363	9740	5326	**7.15**	8377	3.91	3963
ywrO	nitroreductase	3320	6405	6250	1.93	3085	1.88	2930	2838	13591	9194	4.79	**10754**	3.24	6356

Table 5. (continued)

		10 min							30 min						
		Signal Intensity			+End		+Bac		Signal Intensity			+End		+Bac	
Gene	Product	Cont	+End	+Bac	Ratio	Dif	Ratio	Dif	Cont	+End	+Bac	Ratio	Dif	Ratio	Dif
(CtsR regulon)															
ctsR	transcriptional repressor of class III stress genes	4228	11681	8311	2.76	7453	1.97	4083	5399	18104	9339	3.35	**12705**	1.73	3939
mcsA	modulator of CtsR repression	2866	9620	6249	3.36	6755	2.18	3383	4138	17249	8147	4.17	**13110**	1.97	4009
mcsB	modulator of CtsR repression	4741	12225	8747	2.58	7484	1.84	4005	6579	20277	11706	3.08	**13698**	1.78	5127
clpC	class III stress response-related ATPase	6139	13881	10093	2.26	7742	1.64	3954	8049	23396	13970	2.91	**15347**	1.74	5921
clpE	ATP-dependent Clp protease-like	625	5582	2114	**8.94**	4957	3.38	1489	1167	10983	3968	**9.41**	9816	3.40	2801
clpP	ATP-dependent Clp protease proteolytic subunit	12986	18587	15593	1.43	5601	1.20	2607	14617	26917	20586	1.84	**12300**	1.41	5970
(SigI regulon)															
mreBH	cell-shape determining protein	1556	5715	7879	3.67	4158	**5.06**	6322	2355	12209	13452	**5.18**	9854	**5.71**	**11097**
ykpC	function unknown and unique	553	2781	4255	**5.03**	2227	**7.69**	3702	1107	7679	8051	**6.94**	6572	**7.27**	6944
(SigB regulon)															
yvgN	similar to 2,5-diketo-D-gluconic acid reductase	13087	16203	14807	1.24	3116	1.13	1719	15555	32074	27266	2.06	**16519**	1.75	**11711**
(Others)		Cont	End	Bac	E-Ratio	E-Dif	B-Ratio	B-Dif	Cont	End	Bac	E-Ratio2	E-Dif2	B-Ratio2	B-Dif2
ndhF	NADH dehydrogenase subunit 5	359	4280	2914	**11.94**	3922	**8.13**	2556	256	1827	745	**7.14**	1571	2.91	489
ybcC	Probaly fused to YbcD	267	3149	2344	**11.78**	2882	**8.77**	2077	208	1273	531	**6.10**	1064	2.55	323
ybcD	conserved protein	648	5199	3993	**8.02**	4551	**6.16**	3345	300	1935	1041	**6.45**	1635	3.47	741
ybcF	plant-type carbonic anhydrase	741	4247	3793	**5.73**	3506	**5.12**	3052	371	1824	1030	4.91	1452	2.77	659
ydfQ	similar to thioredoxin	122	649	568	**5.32**	527	4.66	446	214	1154	471	**5.38**	940	2.20	257
yheD	putative transcriptional regulator	241	1161	1283	4.82	920	**5.32**	1042	249	1235	1058	4.96	986	4.25	809
yhdC	function unknown and unique	158	1092	1516	**6.90**	934	**9.57**	1358	195	519	272	2.66	323	1.39	77
yuzF	conserved protein	1186	7477	6200	**6.30**	6291	**5.23**	5013	1132	5421	2435	4.79	4288	2.15	1303
fabG	3-ketoacyl-acyl carrier protein reductase	6511	10667	10201	1.64	4156	1.57	3690	7190	17571	13448	2.44	**10381**	1.87	6258
nrgB	nitrogen regulatory PII-like protein	1044	2259	2685	2.16	1216	2.57	1641	1071	8919	5315	**8.33**	7848	4.96	4244
oxdC	acid-induced cytosolic oxalate decarboxylase	291	413	550	1.42	123	1.89	260	791	2411	4093	3.05	1620	**5.17**	3302
yceB	nitro/flavin reductase	605	2652	2283	4.39	2048	3.78	1678	751	5079	2148	**6.76**	4327	2.86	1396
yfhP	negative regulator of yfhQ, fabL, sspE and yfhP	1784	4306	3795	2.41	2522	2.13	2011	1215	6201	3369	**5.10**	4986	2.77	2154
yhzB	conserved protein	1072	2536	2486	2.37	1464	2.32	1414	1041	7859	4030	**7.55**	6818	3.87	2989

Table 5. (continued)

		10 min							30 min						
		Signal Intensity			+End		+Bac		Signal Intensity			+End		+Bac	
Gene	Product	Cont	+End	+Bac	Ratio	Dif	Ratio	Dif	Cont	+End	+Bac	Ratio	Dif	Ratio	Dif
yjiB	cytochrome P450-like enzyme	290	799	799	2.76	509	2.76	510	436	3256	1679	**7.47**	2820	3.85	1244
yjiC	similar to macrolide glycosyltransferase	332	788	828	2.37	456	2.49	496	509	3640	2030	**7.15**	3131	3.99	1521
ykvR	function unknown and unique	261	786	762	3.00	524	2.91	500	339	2194	984	**6.48**	1856	2.91	645
yobE	conserved protein	443	1498	1369	3.38	1054	3.09	925	638	4951	2186	**7.76**	4313	3.42	1548
yoqW	conserved protein in SP-beta	245	942	751	3.84	696	3.06	505	328	3130	1117	**9.54**	2802	3.40	789
yqaB	similar to phage-related protein, SKIN protein	106	409	409	3.87	303	3.87	304	104	726	289	**6.95**	622	2.77	185
yqiK	RNase Z responsible for the maturation of the 3' end of tRNA	714	1683	1621	2.36	969	2.27	906	702	4600	2407	**6.55**	3897	3.43	1705
ytzE	transcriptional regulator	842	2240	1740	2.66	1398	2.07	898	1109	9486	3774	**8.56**	8377	3.40	2665
yveF	function unknown and unique	96	280	278	2.93	184	2.91	183	287	2422	1209	**8.44**	2135	4.22	923
yveG	conserved protein	120	275	271	2.29	155	2.26	151	290	2052	1103	**7.07**	1762	3.80	812
padC	phenolic acid decarboxylase	195	409	434	2.10	215	2.23	239	572	3029	1781	**5.30**	2457	3.11	1209
yvdS	Membrane transporters of cations and cationic drugs	634	2030	1760	3.20	1396	2.78	1126	516	5925	2597	**11.49**	5409	**5.03**	2081
yvdR	SMR-type multidrug efflux transporter	550	1533	1069	2.79	983	1.94	519	604	4726	1993	**7.83**	4123	3.30	1389
ywrF	Conserved protein/domain typically associated withflavoprotein oxygenases, DIM6/NTAB family	1044	2430	2368	2.33	1386	2.27	1324	1548	9319	5269	**6.02**	7772	3.40	3721
yxaH	conserved protein	306	19985	2018	**65.28**	**19679**	**6.59**	1712	452	2124	692	4.70	1672	1.53	240
yxaJ	function unknown and unique	605	3386	485	**5.59**	2780	0.80	-121	1024	486	544	0.47	-538	0.53	-480
gerAA	spore germination protein	158	1010	753	**6.39**	852	4.76	595	214	204	194	0.95	-10	0.91	-20
gerAB	spore germination protein	115	2048	1827	**17.87**	1933	**15.94**	1713	106	162	162	1.53	56	1.53	56
gerAC	spore germination protein	154	1633	1496	**10.63**	1479	**9.74**	1343	134	184	174	1.38	50	1.30	40
pyrK	dihydroorotate dehydrogenase electron transfer subunit	887	5686	4690	**6.41**	4799	**5.29**	3802	1183	843	376	0.71	-339	0.32	-806
pyrD	dihydroorotate oxidase	1571	9375	8039	**5.97**	7804	**5.12**	6468	1982	1399	633	0.71	-583	0.32	-1349
pyrF	orotidine 5'-phosphate decarboxylase	1699	10074	8535	**5.93**	8375	**5.02**	6836	1749	1211	524	0.69	-538	0.30	-1225
pyrE	orotate phosphoribosyltransferase	1899	10677	8837	**5.62**	8777	4.65	6938	2098	1464	719	0.70	-635	0.34	-1380

Table 5. (continued)

		10 min							30 min						
		Signal Intensity			+End		+Bac		Signal Intensity			+End		+Bac	
Gene	Product	Cont	+End	+Bac	Ratio	Dif	Ratio	Dif	Cont	+End	+Bac	Ratio	Dif	Ratio	Dif
ytrA	transcriptional regulator	2324	18842	2188	**8.11**	**16518**	0.94	-136	2105	1876	1077	0.89	-229	0.51	-1029
ytrB	possible acetoin transport system ATP-binding protein	1962	13892	1765	**7.08**	**11929**	0.90	-198	2033	1522	976	0.75	-511	0.48	-1057
ytrC	possible acetoin transport system permease	2085	13006	1628	**6.24**	**10920**	0.78	-457	1471	1314	686	0.89	-157	0.47	-785
ytrD	possible acetoin transport system permease	2218	14762	1955	**6.66**	**12544**	0.88	-263	1750	1575	802	0.90	-174	0.46	-948
ytrE	possible acetoin transport system ATP-binding protein	2163	15915	2042	**7.36**	**13752**	0.94	-121	1999	1870	1055	0.94	-129	0.53	-944
ytrF	possible acetoin transport system substrate binding protein	3026	18794	3104	**6.21**	**15768**	1.03	79	3095	3510	1541	1.13	415	0.50	-1554

a) Genes whose induction ratio is more than 5 or induction level is more than 10,000 units in some experimental condition are listed.

b) Transcription intensity in the presence of enduracidine (+End) and bacitracin (+BaC), and in their absence (Cont), inductio ratio (Ratio) and induction (level) at 10 and 30 min incubation are indicated in each colomn. Values that satisfied the selection criterion are indicated by bold letter.

III.2. Activation of TCSs by enduracidin and bacitracin

Changes in the transcription profiles of five operons containing TCS genes are known to occur in response to cell envelope stress caused by antibiotics (see Figure 4), and are shown in Figure 9. The *lia* operon showed the highest degree of induction by both antibiotics, using both the ratio and level criteria defined above, of the genes listed in Table 5. Transcription of *bceAB* encoding a probable bacitracin exporter was also induced by bacitracin, to a similar level.

Bacitracin has been reported to induce *yvcRS* expression (Mascher et al., 2003). We observed relatively weak induction of *yvcRS* transcription by bacitracin at 10 min after drug addition, and the induction fell at 30 min. Similarly, enduracidin induced *bceAB*, *yvcRS,* and *yxdLM* transcription only at 10 min, although enduracidin also induced transcription of the *bceS* sensor and *bceR* regulator in our experiments, by an unknown mechanism. Involvement of the BceAB transporter in bacitracin resistance has been previously demonstrated (Ohki et al, 2003), and, in the present work, we discovered an important role for the *lia* operon in mediation of enduracidin resistance, as described below. However, any biological significance of transient transcriptional induction of other TCSs remains unclear because inactivation of these TCSs has resulted in no obvious phenotypic change, at least in the reports that have appeared to date. Sensor kinases in the TCSs are characterized by unique N-terminal input domains, consisting of only two transmembrane helices with a very short periplasmic linker sequence (Mascher et al., 2003), and BceRS has been proposed to respond to bacitracin transport by BceAB rather than to the extracellular presence of the drug *per se* (Bernard et al., 2007). Thus, both systems are classified as intramembrane-sensing sensor kinases (Mascher, 2006). Transient activation of YvcPQ and YedJK TCSs by

both antibiotics, and the BceRS TCS by enduracidin, might result from transient distortions of membrane structure by the action of antibiotics.

Additionally, HtrA and HtrB proteases under the control of the CssRS TCS which senses abnormal membrane proteins (Darmon et al., 2002) were induced by antibiotics, as discussed below

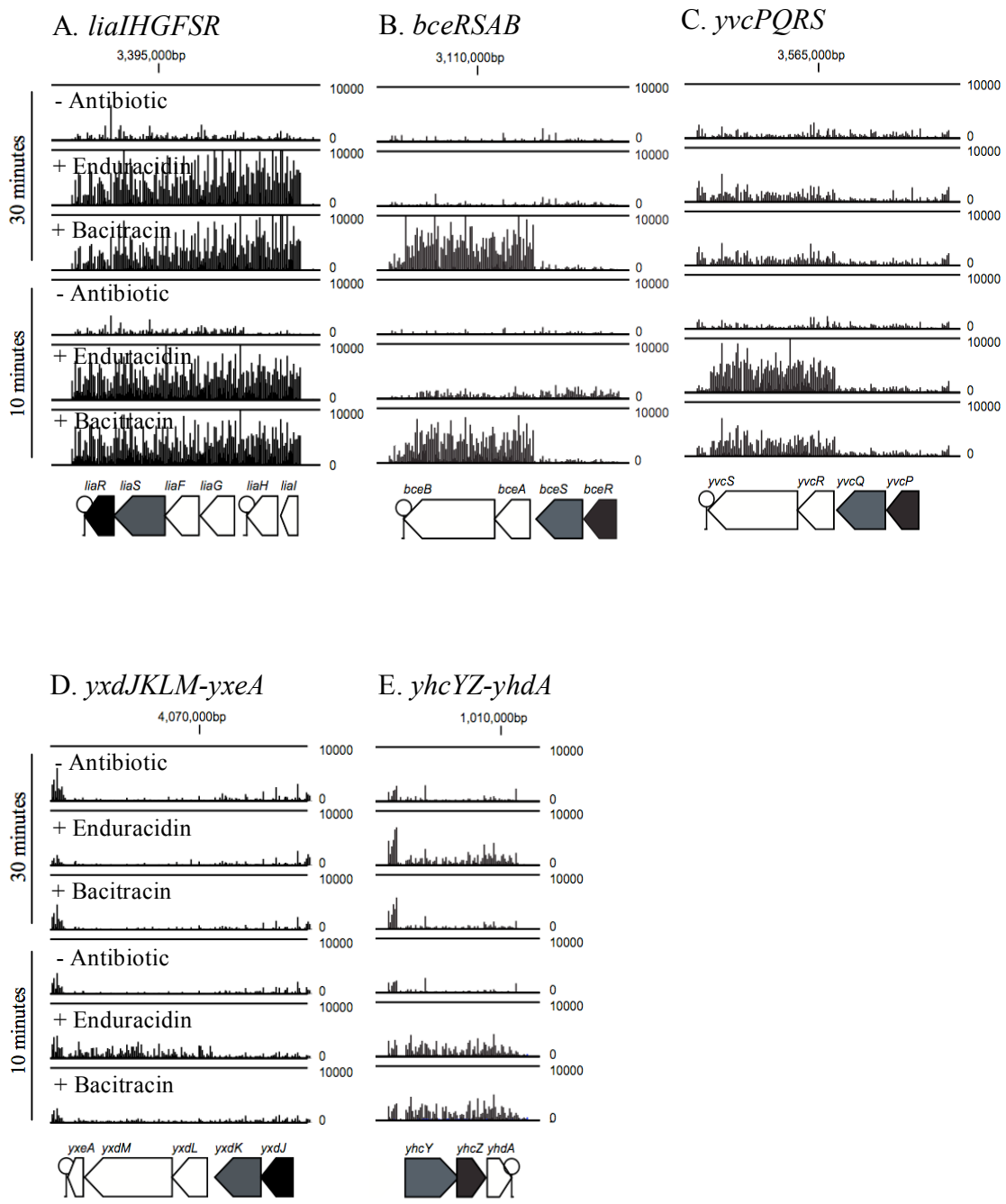

Figure 9. Transcriptional profiles of *liaRS*, *bceRS*, *yvcPQ*, *yxdJK*, and *yhcZY* operons. Transcriptional profiles of *liaRS* (A), *bceRS* (B), *yvcPQ* (C), *yxdJK* (D), and *yhcZY* (E) operons are shown. Transcriptional signals for each probe are indicated by vertical bars at appropriate genomic coordinates. At the bottom, gene arrangements are schematically shown.

III.3. Northern blot analysis of induction of the *lia* operon expression by enduracidin

We also performed northern blot analysis to further analyze the induction of *lia* operon expression by 30 min incubation with 0.025 μg/ml enduracidin in wild type 168 cells and cells inactivated for each gene in *lia* operon (Figure 10). Multiple transcripts were detected in wild type and *liaF*, *liaG*, *liaH* or *liaI* inactivated cells treated with enduracidin. In contrast, no transcripts were detected in *liaR* or *liaS* inactivated cells, demonstrating clearly that the induction of *lia* operon expression is completely dependent on LiaS HK and LiaR RR. Interestingly, the *lia* operon was highly transcribed even in the absence of enduracidin in the *liaF* mutant, suggesting that LiaF is a negative regulator of the LiaRS activity. The similar phenomenon was found by Mascher et al. (2004) using the *lacZ* reporter fused to the *lia* operon promoter. Among transcripts detected, the strong band at around 1 kb would correspond to *liaIH* polycistronic transcripts, as it is detected by *liaI* and *liaH* probes and not by *liaF* and *liaG* probe. Consistently its size was reduced in *liaI* and *liaH* mutants. In addition, all probes detected multiple transcripts extending to downstream of *liaIH*, although it is not clear they arose by premature termination or degradation of the *liaIHGFSR* transcript. These high-molecular transcripts seemed to be reduced in the *liaH* mutant with unknown reason.

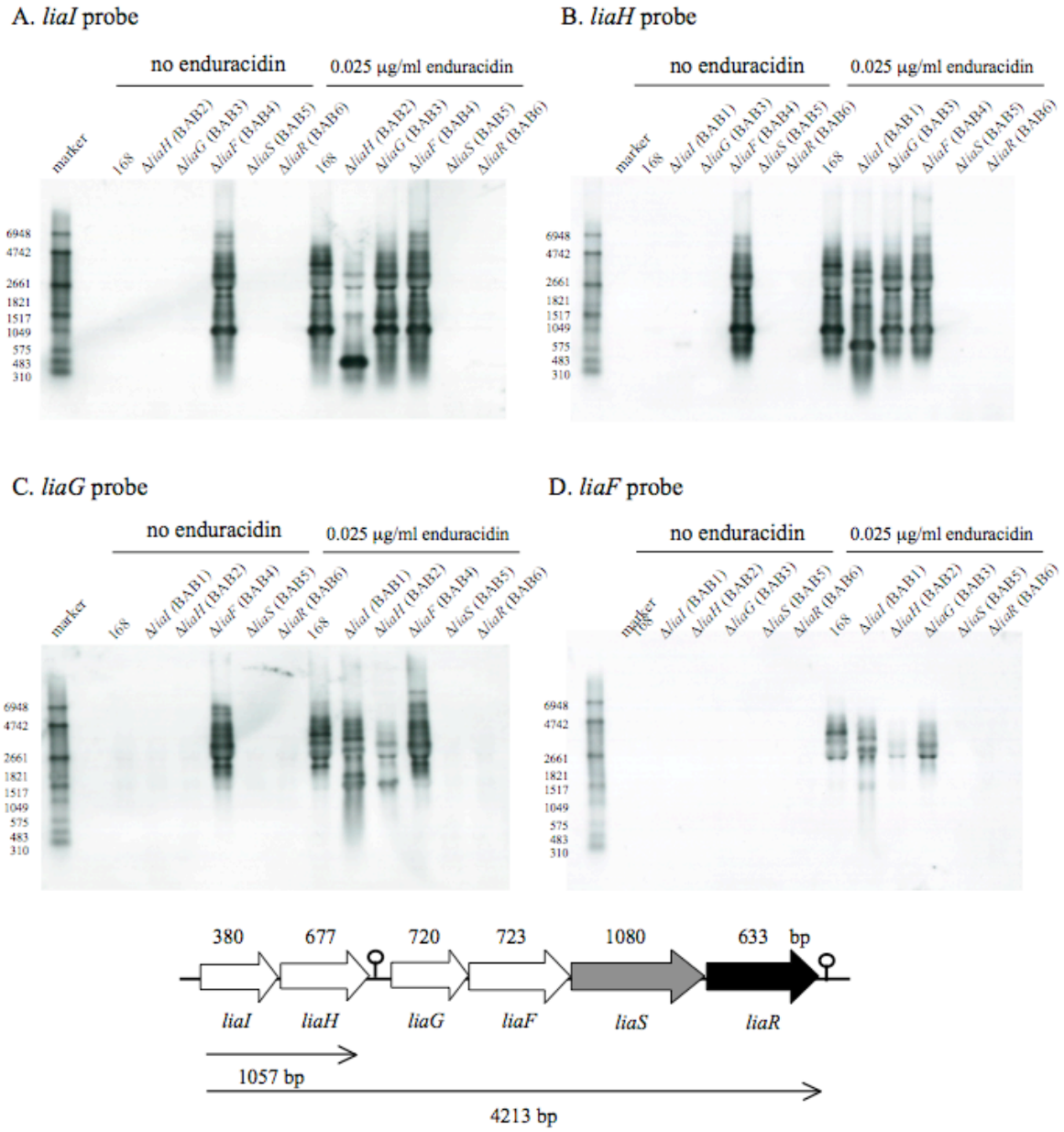

Figure 10. Northern blot analysis to the *lia* operon expression in the presence and absence of enduracidin. Each lane contains 1 µg total RNA purified from wild type 168 cells and cells inactivated for each gene in *lia* operon. Probes used to detect transcripts were indicated above the blots. Marker sizes are indicated on the left of the blots.

III.4. Identification of the LiaR binding sequence in the *lia* operon promoter

We examined direct binding of LiaR to the *lia* operon promoter to activate transcription form it. To this end, we first determined the transcription start site by primer extension analysis using RNA purified from BAB16 cells, over-producing LiaR under the control of an IPTG inducible promoter on the multi-copy pDG148 plasmid (Kobayashi et al. 2001). The result shown in Figure 11 clearly indicated that transcription of the *lia* operon initiates at an A residue located 23 nucleotides upstream of the *liaI* initiation codon. We detected possible -10 consensus sequence for σA, TATAAG, at 9 bp upstream of the transcription start site. On the other hand, no clear -35 sequence was observed at the appropriate position from the -10 sequence, which is typical of promoters regulated by transcriptional activators.

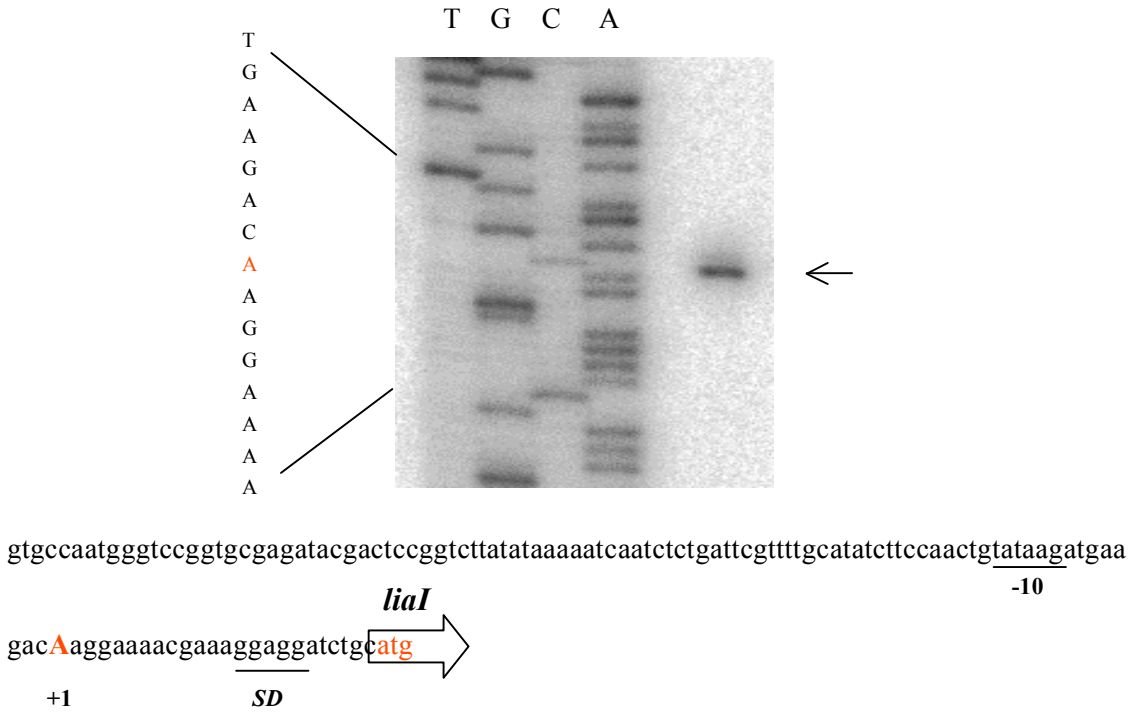

Figure 11. Determination of the transcription start site of *lia* operon by primer extension. Total RNA was prepared from BAB16 cells harbouring pDG148-*liaR* grown in LB medium with IPTG and used for primer extension analyses. Sequencing ladders (lanes T, G, C and A) were generated with the primer used for the reverse transcriptase reaction. The sequence and transcriptional start site (indicated by an arrow) are shown.

To analyze the *cis-acting* sequence required for induction of *lia* operon, we performed the β-galactosidase assay based on promoter dissection-*lacZ* approach. Fragments starting at +82 (inside of *liaI*) bp relative to transcription start site of the *lia* operon and extending to different length upstream of transcriptional start site were used to generate *lacZ* reporter fusion in the *amyE* locus of the genome as shown in Figure 12. The β-galactosidase (LacZ) activity was significantly induced by enduracidin in cells containing constructs that included 97 bp of the upstream region. Enduracidin-induced LacZ activity was reduced by deletion of -78 to -98 bp sequence, and completely abolished by further deletion to -57 bp (Figure 12). These results indicated that sequence between -98 to -57 bp is essential for induction of transcription by LiaR, and we identified a 6-nucleotide direct repeat TCCGGT with a 12-nucleotide spacing locating at -84 to -61 bp as a candidate for DNA binding site of LiaR. The same repeat sequence was reported as *cis*-acting sequence required for induction of *lia* operon by LiaR by Mascher et al. (2004), although direct binding of LiaR to it has not been examined. Finally, we performed the mobility gel shift assay to demonstrate the LiaR binding to the promoter region of the *lia* operon promoter in vitro. LiaR fused to GST were over-expressed in *E. coli*, and purified to near homogeneity using the affinity column (Figure 13A). Results of mobility gel shift assay shown in Figure 13B indicated that LiaR-GST indeed bound fragment DNA II containing direct repeat sequence TCCGGT-12nt-TCCGGT. No binding to fragment III suggests that sequence flanking the repeat one required for the efficiency of LiaR binding.

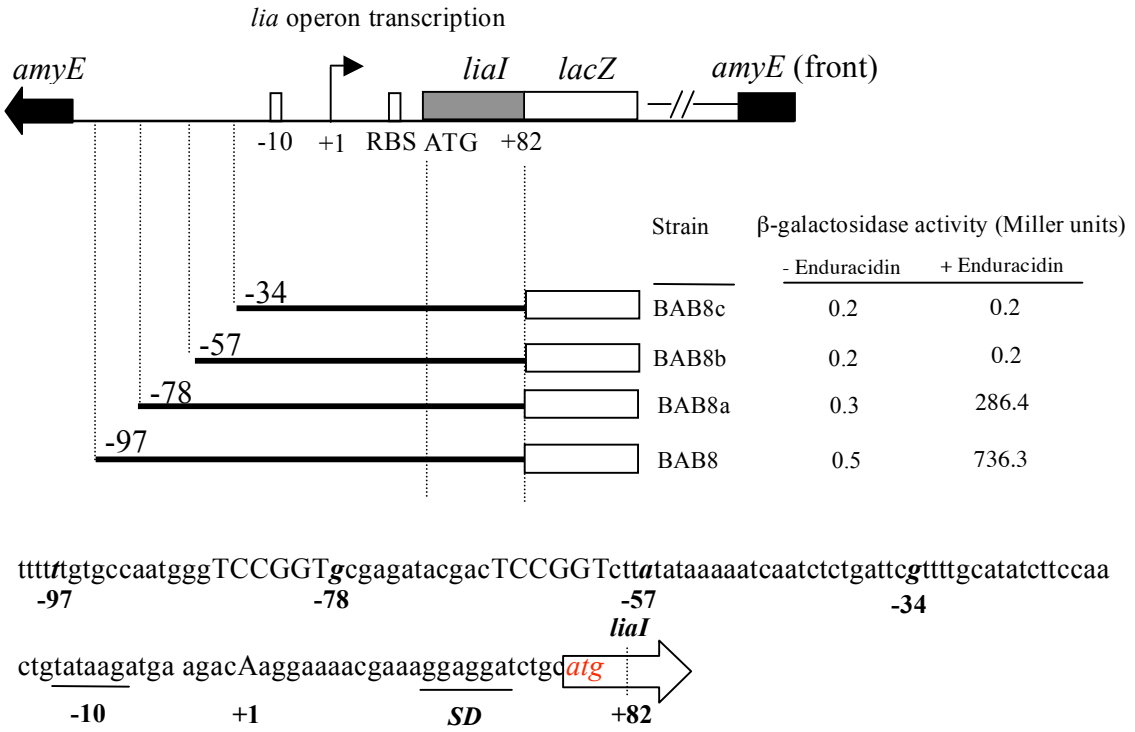

Figure 12. Determination *cis-acting* sequence required for induction of *lia* operon by β-galactosidase assay. Transcriptional fusion strains harboring various *lia* operon promoter regions transcriptionally fused to the *lacZ* reporter gene at the *amyE* locus on the *B. subtilis* genome were constructed, as shown schematically. β-Galactosidase activities are shown for strains cultivated in the absence (–) or presence of enduracidin (+).

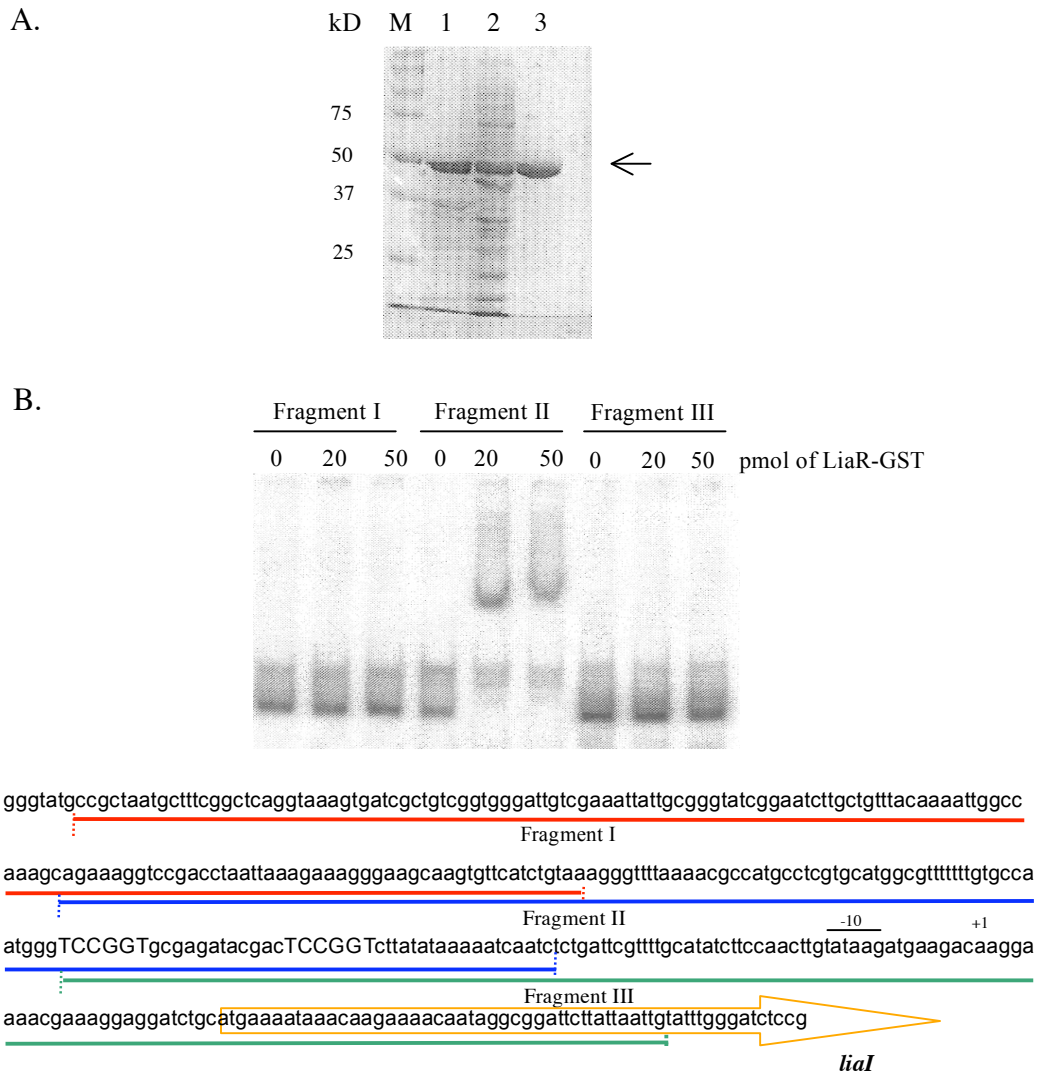

Figure 13. Mobility gel shift assay to demonstrate the LiaR binding to upstream of the *lia* operon promoter. (A) SDS-PAGE profile of purified LiaR-GST. Lane M, 1, 2, and 3 contain molecular weight standard (Bio-Rad), protein in pellet, supernatan, and eluate fraction respectively. LiaR-GST is indicated by black arrow. (B) LiaR-GST protein was incubated with 24.25 pmol DNA fragment I, II, and III, and binding to them was examined by 5% polyacrylamide gel electrophoresis. Sequences of probe DNA fragments are shown in the bottom.

III.5. ChAP-chip analysis of the LiaR binding site(s) on the *B. subtilis* genome

Expression of *yhcZY-yhdA* was also initially induced by the two antibiotics, and the expression has been suggested to be under the control of LiaRS (Jordan et al.,

2006). To examine this possibility, we determined the LiaR binding site(s) on the genome using a modified ChIP-chip method (Ishikawa et al., 2007), employing a strain harboring the *liaR* gene fused with a 12x-histidine coding sequence at the 3'-terminus. The results shown in Figure 14A (see also Supplementary Figure 2) demonstrate LiaR binding only in the promoter region of the *lia* operon, at 30 min after addition of enduracidin, at which time *lia* operon transcription was maximally induced. Additionally, we sought enrichment of the promoter sequence of the *yhcZY* operon in the ChAP fraction by quantitative PCR, at both 10 and 30 min after addition of the drug, with negative results (Figure 14B). Although LiaR-dependent expression of the *yhcZY* operon has been demonstrated in LiaF-inactivated cells, in which LiaRS is constitutively activated (Jordan et al., 2006), our results suggest that *yhcZY-yhdA* expression would also be transiently induced by initial distortion of the cell membrane, independent of LiaRS status in wild-type cells.

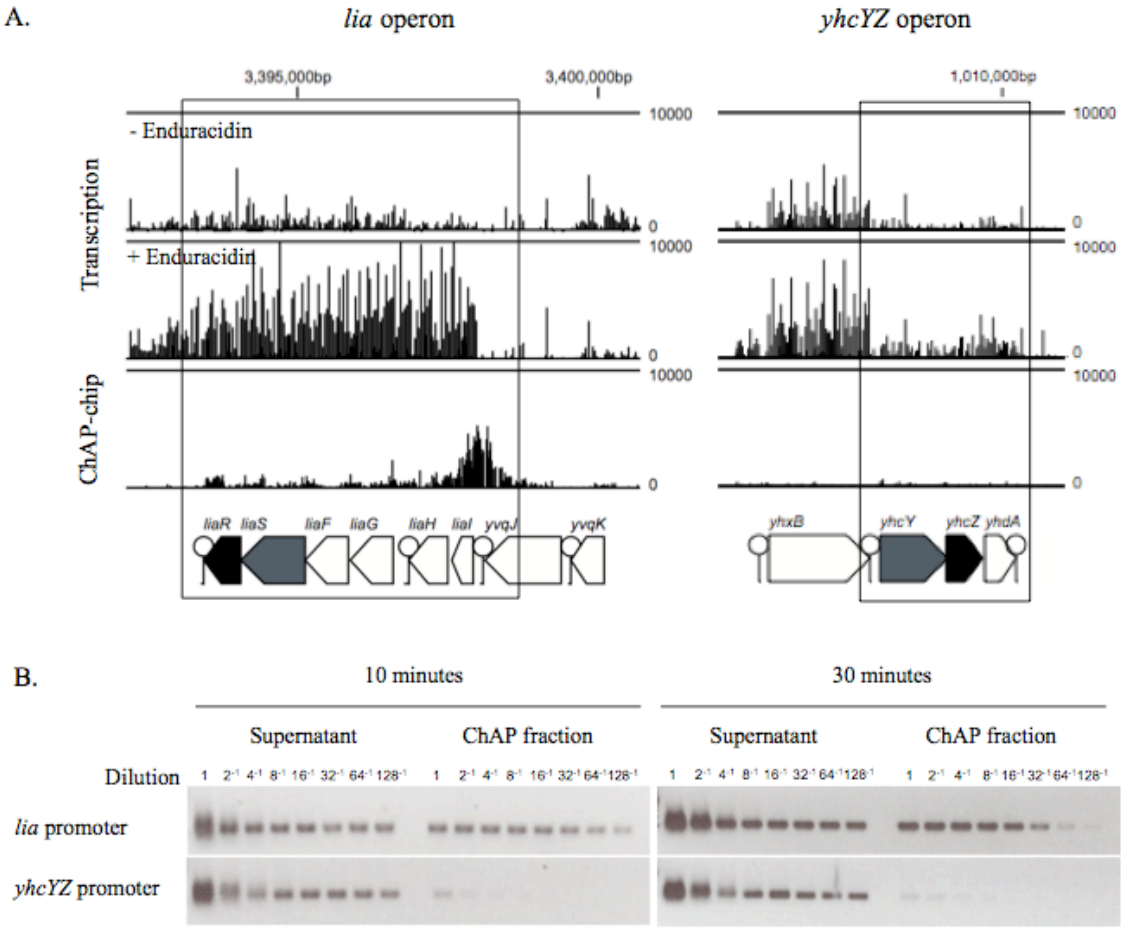

Figure 14. LiaR binding profile on the *B. subtilis* genome. (A) LiaR binding signals in *lia* and *yhcYZ* operons 30 min after the addition of enduracidin are shown by vertical bars at appropriate genome coordinates, together with transcriptional signals in enduracidin-treated and control cells. (B) DNA fragments that co-purified with LiaR and those in the supernatant fraction used for affinity purification were used as templates for PCR amplification. Quantitative PCR was performed with primers specific for the promoter sequences of the *lia* and *yhcZY* operons, using serial dilutions of DNA fragments, as indicated.

III.6. Involvement of Lia proteins in enduracidin resistance

Expression of the *lia* operon is strongly induced by antibiotics that interfere with the lipid II cycle of peptidoglycan biosynthesis, although *liaRS* inactivation did not affect the sensitivity of *B. subtilis* cells to such antibiotics (Pietiainen et al., 2005). A recent study disclosed that daptomycin, which is supposed to disrupt cell membrane structure directly, also induced expression of the *lia* operon, and that deletion of the

liaH gene led to a three-fold increase in susceptibility to daptomycin (Hachmann et al., 2009). We created clean deletion mutants of each gene in the *lia* operon to avoid any polar effect of selection marker fragments on transcription of downstream genes, and examined their sensitivities to enduracidin and bacitracin by spotting serial dilutions of wild-type and mutant cells onto LB agar plates containing 0.005 or 0.01 μg/mL enduracidin and 50 μg/mL bacitracin (Figure 15). Growth inhibition by enduracidin was more severe on agar plates, compared to liquid medium, for an unknown reason. We included in analysis a *bcrC* mutant known to be sensitive to bacitracin, as a control. The data of Figure 15 show that inactivation of *bcrC* increased the bacitracin sensitivity of *B. subtilis* cells, but inactivation of the *lia* operon did not affect sensitivity. Notably, we found that inactivation of *liaR* or *liaS* resulted in an enduracidin-hypersensitive phenotype. Furthermore, we demonstrated that not only the LiaH protein (homologous to the *E. coli* PspA protein), but also the membrane protein LiaI co-induced with LiaH, played an important role in mediation of enduracidin resistance. The LiaR binding specificity shown in Figure 14 suggests that the increased enduracidin sensitivity of *liaS* and *liaR* mutants was attributable to a failure to induce *liaIH* expression. It was interesting to note that inactivation of *liaG* had no effect on enduracidin resistance, but *liaF* inactivation, that induced constitutive expression of the *liaRS* operon (Jordan et al., 2006), led to increased resistance to the antibiotic.

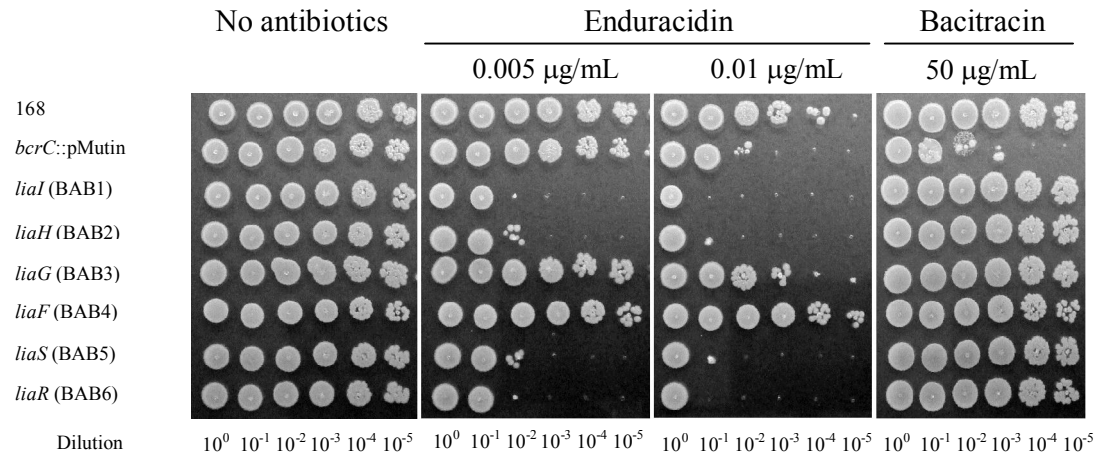

Figure 15. Effect of inactivation of genes of the *lia* operon on enduracidin and bacitracin resistance. Overnight cultures of wild-type and mutant *B. subtilis* cells in LB medium, grown at 37˚C, were diluted to an OD_{600} value of 1.0, and further serially diluted as indicated. Next, 2 μL aliquots of cells were spotted onto LB agar plates with or without antibiotics, and incubated overnight at 37˚C.

The *E. coli* homolog of LiaH, PspA, is induced under membrane stress conditions and is proposed to bind to membrane phospholipids to suppress proton leakage (Kobayashi et al., 2007). Although the mechanisms of action of enduracidin and daptomycin are different, the antibiotics are structurally related lipodepsipeptides produced by *Streptomyces* species. The primary mode of action of daptomycin is considered to be direct disruption of the functional integrity of the cytoplasmic membrane to induce depolarization. However, inhibition of peptidoglycan biosynthesis, either directly or indirectly, is also thought to be a feature of daptomycin action (Muthaiyan et al., 2008). Conversely, although the primary target of enduracidin is the transglycosylation step of peptidoglycan biosynthesis, the drug might also induce a distortion of the membrane structure similar to that caused by daptomycin, which needs to be counteracted by LiaH to re-establish membrane functionality. The *lia* locus of *Bacillus* species consists of five core genes, homologs of *liaIHFSR*, whereas only *liaFSR*-homologous genes are conserved in more distantly related firmicutes bacteria

(Jordan, 2008). Although the sensitivity of the *liaI* mutant to daptomycin has not been examined, LiaI might play an important role in supporting LiaH function in *Bacillus* species.

Additionally, inactivation of *bcrC* seemed to reduce enduracidin resistance at the higher concetration. Although BcrC was first identified as a bacitracin-resistance determinant, subsequent studies demonstrated that BcrC has an undecaprenyl pyrophosphate (UPP) phosphatase activity (Bernard et al., 2005). The *bcrC* mutant has been reported to be sensitive to paraquat stress (Cao et al., 2005), in addition to bacitracin. Thus, inactivation of *bcrC* will influence resistance to broader stresses through unknown changes in cell envelope structure.

III.7. Involvement of ECF sigma factors in enduracidin resistance

Among the 129 genes listed in Table 6, we found that 33 belonged to the SigM regulon reported by Eiamphungporn and Helmann (2008). It is known that activated ECF sigma factors auto-activate their own expression, and the self-activation of *sigM* transcription was evident in our analysis. Generally, transcription induction of the SigM regulon by enduracidin and bacitracin was similar at 10 min after drug addition, but further induction at 30 min was observed only in the presence of enduracidin. Transcriptional levels of seven ECF sigma factors encoded in the *B. subtilis* genome are summarized in Table 6. Although *sigV* is included in Table 6, the *sigV* transcriptional levels were much lower than those of *sigM.* The transcriptional levels of *sigW* were highest among the ECF sigma factor genes assessed under our growth conditions, but the levels were not affected by either antibiotic. Transcription of *sigX* was also relatively high, although the level fell after 30 min incubation with antibiotics.

Accordingly, we could not identify genes that might be upregulated by SigW or SigX after the addition of either antibiotic.

Table 6. Transcrition levels of ECF sigma factor genes

	10 min							30 min						
	Signal Intensity			+End		+Bac		Signal Intensity			+End		+Bac	
Gene	Cont	+End	+Bac	Ratio	Dif	Ratio	Dif	Cont	+End	+Bac	Ratio	Dif	Ratio	Dif
sigI	1861	7028	6617	3.78	5167	3.56	4757	2455	7393	5867	3.01	4938	2.39	3412
sigM	4286	16957	16028	3.96	**12672**	3.74	**11743**	3323	18432	12318	**5.55**	**15109**	3.71	8995
sigV	436	1637	1605	3.75	1200	3.68	1168	353	3680	1881	**10.44**	3327	**5.34**	1528
sigW	20055	24102	23105	1.20	4047	1.15	3050	15323	17708	16067	1.16	2384	1.05	743
sigX	5263	4853	5021	0.92	-410	0.95	-242	5384	2861	4712	0.53	-2523	0.88	-672
sigY	864	1673	1525	1.94	809	1.76	661	1401	3660	2023	2.61	2259	1.44	622
sigZ	265	281	328	1.06	16	1.24	63	437	574	512	1.31	137	1.17	75
ylaC	669	1300	1477	1.94	631	2.21	808	620	988	837	1.59	368	1.35	217

a) Transcription levels of ECF sigma factor genes are indicated as in Table 5.

Next, we examined the effect of inactivation of each gene encoding an ECF sigma factor on sensitivity to enduracidin and bacitracin. The data of Figure 16 indicate that single inactivations of ECF sigma genes did not affect bacitracin sensitivity, as reported previously (Mascher et al., 2003). The *bcrC* gene is known to be the target of SigM, but *bcrC* has been suggested to be a target of SigW, SigX, and SigV. These latter sigma factors might compensate for inactivation of SigM function. Consistent with stronger induction of the SigM regulon in the presence of enduracidin, inactivation of *sigM* increased sensitivity to the antibiotic, to a level similar to that seen in the *liaR* mutant (Figure 16), suggesting that some gene(s) specifically induced by SigM also plays an important role in enduracidin resistance. However, the decreased enduracidin resistance displayed by the *sigX* mutant at the higher concentration was unexpected. In contrast, change in bacitracin resistance was not observed on LB plates containing 100 or 150 μg/mL bacitracin (data not shown). Transcriptomic analysis of the *sigM* and *sigX* mutants, seeking to identify genes specifically regulated by these sigma factors in the presence of enduracidin, are required to clarify the molecular mechanisms of involvement of the factors in enduracidin resistance.

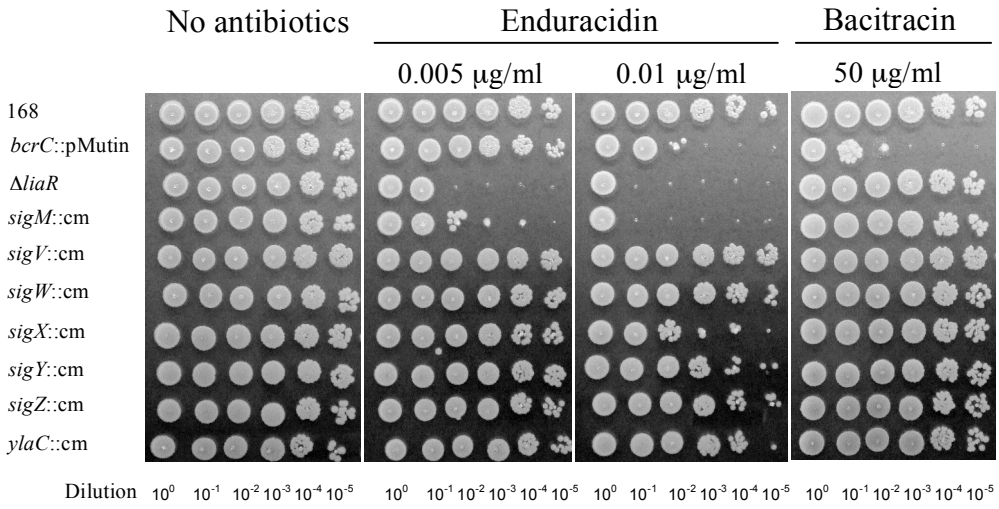

Figure 16. Effect of inactivation of ECF sigma factors on enduracidin and bacitracin resistance. The sensitivities of wild-type and mutant cells to enduracidin and bacitracin were examined as described in the legend to Figure 15.

III.8. Induction of the Spx regulon

The Spx protein is a unique regulator interacting with the α subunit of RNA polymerase and reorganizing gene expression in response to thiol-oxidative stress (Nakano et al., 2003; Reyes et al., 2008). The Spx protein represses expression of target genes by blocking interaction of gene activators with the α subunit of RNA polymerase. The Spx protein also activates expression of a number of genes by recruiting RNA polymerase through recognition of a motif located upstream of promoters (Reyes et al., 2008). An oxidized form of Spx, in which the N-terminal CXXC motif is in the disulfide state, is essential for formation of the active Spx-RNA polymerase complex, and *spx* expression is under complex regulation, including activation by SigM. Indeed, Spx expression was induced by enduracidin and bacitracin in our analysis. Furthermore, we found that 21 of the genes listed in Table 2 were activated by Spx (Nakano et al., 2003), strongly suggesting that thiol-oxidative stress

is induced by enduracidin and bacitracin challenge. In addition to induction of the CssRS regulon (Damon et al., 2002) as described above, we observed the induction of proteases belonging to the CtsR regulon that is involved in protein quality control in the cytoplasm (Miethke et al., 2006). Activation of these genes might result from damage to proteins caused by thiol-oxidative stress. Supporting this idea, co-induction of CtsR and Spx regulons has been observed after treatment of *B. subtilis* cells with 6-brom-2-vinyl-chroman-4-on (chromanon), 2-methylhydroquinone (2-MHQ), and salicylic acid, all of which would cause protein damage (Duy et al., 2007; Nguyen et al., 2007). The *B. subtilis sigI* gene encodes an alternative sigma factor of the sigma70 family, and is involved in heat-shock resistance (Tseng and Shaw, 2008). The *mreBH* gene listed in Table 2 was recently found to be the target of SigI, and the transcriptional level of *sigI* was increased by the two antibiotics, although the levels did not satisfy our criteria for extraction of genes for inclusion in Table 2. The phenomena discussed above may also be related to damage to proteins.

Although induction of thiol-oxidative stress by antibiotics targeting the cell envelope has not been previously reported, obvious TrxA induction has been observed in daptomycin- and friulimicin B-treated cells (Wecke et al, 2009), suggesting that thiol-oxidative stress induction might be general under cell wall stress conditions induced by antibiotics. However, inactivation of *spx* did not affect the sensitivity of *B. subtilis* cells to enduracidin and bacitracin (data not shown). Further studies are necessary to clarify the relationships between thiol-oxidative stress induction and actions of antibiotics interfering with cell envelope integrity.

III.9. Induction of other genes

The list of genes upregulated by enduracidin and/or bacitracin (Table 5) contains about 40 additional genes whose induction mechanisms and biological significance in relation to drug resistance remain unclear. Induction profiles of a number of these genes were similar to those of genes of the SigM or Spx regulons, and some of the "other" genes may be under the control of SigM or Spx. Several genes were transiently induced at 10 min after drug addition. Induction of the *gerA* operon by bacitracin was observed by Mascher and colleagues (2003), and *gerA* was also induced by daptomycin (Hachmann et al., 2009). Mascher and co-workers (2003) observed induction of the *ytrABCDEF* operon by bacitracin, whereas we found induction only in the presence of enduracidin. Induction of these genes may be very sensitive to subtle changes in cell envelope structure, as is the case with some of the TCSs discussed above. Further experiments are necessary to evaluate the biological significance of these "other" genes in relation to antibiotic resistance.

Mascher and colleagues (2003) additionally proposed that the SigB-dependent general stress regulon (Hecker and Volker, 2001) is activated by bacitracin. However, expression of genes listed as belonging to the *sigB* regulon by Hoper and colleagues (2005) was not greatly affected in our experiments, except for *yvgN*, suggesting that the growth conditions used by Mascher et al. more severely affected *B. subtilis* cells than did our experimental conditions.

III. 10. Genes significantly downregulated by enduracidin and/or bacitracin

Although previous transcriptomic studies of transcriptional responses to antibiotics focused on upregulated genes to identify genes that would involve in antibiotics resistance, we additionally examined significantly downregulated genes by antibiotics treatment. We identified 85 genes whose transcription level was reduced more than five hold in ratio and/or more than 10,000 units in level by enduracidin and/or bacitracin compared to control cells (Table 7). Interestingly, 39 genes among them encode transporters, mainly for sugars, nucleotides, and ions, or belong to operons encoding them. In addition, products of other 14 genes are also associated with cell membrane, including ATP sythetase and cytochrome aa3 quinol oxidase complexes. Downregulation of these genes was observed essentially both in enduracidin- and bacitracin-treated cells, suggesting that these changes would be consequences of changes in metabolite flow due to perturbation of membrane structure by antibiotics. In addition, downregulation of genes encoding translational machinery (8 genes) and metabolic enzymes (10 genes) occurred mainly in cells treated with enduracidin for 30 minutes. In sum, in agreement with general view, we could not clearly detect candidate genes that might be involved in antibiotics resistance, among significantly downregulated genes.

Table 7. Genes significantly downregulated by enduracidin and/or bacitracin

		10 min							30 min						
		Signal Intensity			+End		+Bac		Signal Intensity			+End		+Bac	
Gene	Product	Cont	+End	+Bac	Ratio	Dif	Ratio	Dif	Cont	+End	+Bac	Ratio	Dif	Ratio	Dif
(transporter genes and opeons; 39 genes)															
dctP	C4-dicarboxylate transport protein	7524	5127	7589	0.68	-2397	1.01	65	19310	7270	7088	0.38	**-12041**	0.37	**-12222**
glpQ	glycerophosphoryl diester phosphodiesterase	15721	5379	6534	0.34	**-10342**	0.42	-9187	10606	1927	1184	**0.18**	-8679	**0.11**	-9422
glpT	glycerol-3-phosphate permease	19098	5914	8120	0.31	**-13184**	0.43	**-10977**	12838	1787	1042	**0.14**	**-11051**	**0.08**	**-11796**
lctP	L-lactate permease	3675	587	1000	**0.16**	-3088	0.27	-2675	665	379	509	0.57	-286	0.77	-156
licA	PTS lichenan-specific enzyme IIA component	946	476	655	0.50	-470	0.69	-291	5527	1268	857	0.23	-4259	**0.16**	-4669
licB	PTS lichenan-specific enzyme IIB component	298	194	272	0.65	-104	0.91	-26	1415	321	230	0.23	-1094	**0.16**	-1185
licC	PTS lichenan-specific enzyme IIC component	744	412	580	0.55	-332	0.78	-164	3676	733	569	**0.20**	-2942	**0.15**	-3107
licH	6-phospho-beta-glucosidase	985	534	702	0.54	-451	0.71	-283	6024	1221	973	**0.20**	-4803	**0.16**	-5051
rbsA	D-ribose transport system ATP-binding protein	11715	4412	7667	0.38	-7303	0.65	-4048	26341	17582	9861	0.67	-8759	0.37	**-16480**
rbsB	D-ribose transport system substrate-binding protein	10901	3334	5639	0.31	-7566	0.52	-5262	28323	17503	11175	0.62	**-10820**	0.39	**-17149**
rbsC	D-ribose transport system permease protein	7264	2031	3633	0.28	-5234	0.50	-3631	21282	11403	6379	0.54	-9879	0.30	**-14903**
rbsD	D-ribose transport system permease protein	7363	2521	5330	0.34	-4842	0.72	-2033	18377	11749	6232	0.64	-6628	0.34	**-12145**
rbsK	ribokinase	12625	5725	9035	0.45	-6901	0.72	-3591	23646	16682	10815	0.71	-6964	0.46	**-12832**
rbsR	transcriptional repressor of the rbs operon	14646	6598	10677	0.45	-8048	0.73	-3969	25987	18792	12824	0.72	-7194	0.49	**-13163**
ywsB	conserved protein	10671	3549	5145	0.33	-7122	0.48	-5526	28692	17666	12298	0.62	**-11026**	0.43	**-16394**
pbuG	hypoxanthine/guanine permease	8012	2909	3333	0.36	-5103	0.42	-4679	7052	917	2740	**0.13**	-6135	0.39	-4312
ptsG	PTS glucose-specific enzyme IICBA component	16653	11647	12400	0.70	-5006	0.74	-4253	20063	3461	5250	**0.17**	**-16602**	0.26	**-14813**

Table 7. (continued)

		10 min							30 min						
		Signal Intensity			+End		+Bac		Signal Intensity			+End		+Bac	
Gene	**Product**	**Cont**	**+End**	**+Bac**	**Ratio**	**Dif**	**Ratio**	**Dif**	**Cont**	**+End**	**+Bac**	**Ratio**	**Dif**	**Ratio**	**Dif**
pyrAA	carbamoyl-phosphate synthase small chain	1451	5683	4672	3.92	4232	3.22	3221	3977	1031	433	0.26	-2946	**0.11**	-3544
pyrB	aspartate carbamoyltransferase catalytic chain	879	2160	1981	2.46	1281	2.25	1102	4104	493	338	**0.12**	-3611	**0.08**	-3766
pyrC	dihydroorotase	984	2885	2438	2.93	1901	2.48	1453	3830	587	322	**0.15**	-3243	**0.08**	-3508
pyrP	uracil permease	5293	3129	2706	0.59	-2164	0.51	-2587	11362	818	1144	**0.07**	**-10544**	**0.10**	**-10218**
pyrR	transcriptional attenuator of the pyrimidine operon / uracil phosphoribosyltransferase	10435	3219	2609	0.31	-7216	0.25	-7826	14364	1711	3608	**0.12**	**-12652**	0.25	**-10756**
sunT	sublancin 168 lantibiotic transporter	590	130	189	0.22	-459	0.32	-400	2163	140	667	**0.06**	-2023	0.31	-1497
treP	PTS trehalose-specific enzyme IIBC component	21183	14364	17311	0.68	-6818	0.82	-3872	28611	17137	17137	0.60	**-11474**	0.60	**-11474**
xynP	beta xyloside H+-symporter	119	93	119	0.78	-26	1.01	1	720	118	99	**0.16**	-602	**0.14**	-620
ycnI	conserved membrane protein	11524	2231	2048	**0.19**	-9292	**0.18**	-9475	4269	3095	5061	0.73	-1174	1.19	792
ycnJ	probable copper export protein	10942	2043	1997	**0.19**	-8899	**0.18**	-8945	4061	3094	4865	0.76	-967	1.20	804
ycnK	probable transcriptional regulator	15142	3068	2915	**0.20**	**-12074**	**0.19**	**-12226**	5005	2965	5491	0.59	-2040	1.10	485
ydhM	PTS oligo-beta-mannoside-specific enzyme IIB component	3249	1659	2386	0.51	-1590	0.73	-863	8912	1963	1666	0.22	-6949	**0.19**	-7246
ydhN	PTS oligo-beta-mannoside-specific enzyme IIA componen	912	399	619	0.44	-513	0.68	-293	2682	540	437	**0.20**	-2142	**0.16**	-2245
ydhO	PTS oligo-beta-mannoside-specific enzyme IIC component	1419	604	915	0.43	-816	0.64	-504	3905	763	678	**0.20**	-3141	**0.17**	-3227
ydhP	phospho-beta-glucosidase	1252	597	833	0.48	-655	0.66	-420	3756	767	692	**0.20**	-2989	**0.18**	-3063
yfiY	arthrobactin transport system substrate-binding protein, Fur regulon	9444	7734	6422	0.82	-1710	0.68	-3022	16068	4650	8413	0.29	**-11418**	0.52	-7656
yfmC	ferric citrate transport system substrate-binding protein, Fur regulon	10197	11716	8862	1.15	1519	0.87	-1335	15245	5043	6805	0.33	**-10202**	0.45	-8440
yhfQ	putative iron(III) dicitrate transport system substrate-binding protein	10022	9469	6871	0.94	-553	0.69	-3152	14826	4164	9002	0.28	**-10662**	0.61	-5824

Table 7. (continued)

		10 min							30 min						
		Signal Intensity			+End		+Bac		Signal Intensity			+End		+Bac	
Gene	**Product**	**Cont**	**+End**	**+Bac**	**Ratio**	**Dif**	**Ratio**	**Dif**	**Cont**	**+End**	**+Bac**	**Ratio**	**Dif**	**Ratio**	**Dif**
ykbA	probable amino acid permease	2229	897	1108	0.40	-1332	0.50	-1121	2301	466	1109	**0.20**	-1834	0.48	-1192
yutK	probable nucleoside uptake transporter	4828	2318	2678	0.48	-2510	0.55	-2150	4203	784	1490	**0.19**	-3419	0.35	-2713
yvfM	putative maltose/maltodextrin transport system permease protein	419	212	269	0.51	-206	0.64	-150	1337	345	266	0.26	-992	**0.20**	-1071
yxeB	ferrioxamine transport system substrate-binding protein, Fur regulon	8355	10163	5269	1.22	1808	0.63	-3086	14436	3870	8528	0.27	**-10567**	0.59	-5909
pyrR	transcriptional attenuator of the pyrimidine operon / uracil phosphoribosyltransferase	10435	3219	2609	0.31	-7216	0.25	-7826	14364	1711	3608	**0.12**	**-12652**	0.25	**-10756**
sunT	sublancin 168 lantibiotic transporter	590	130	189	0.22	-459	0.32	-400	2163	140	667	**0.06**	-2023	0.31	-1497
treP	PTS trehalose-specific enzyme IIBC component	21183	14364	17311	0.68	-6818	0.82	-3872	28611	17137	17137	0.60	**-11474**	0.60	**-11474**
xynP	beta xyloside H+-symporter	119	93	119	0.78	-26	1.01	1	720	118	99	**0.16**	-602	**0.14**	-620
ycnI	conserved membrane protein	11524	2231	2048	**0.19**	-9292	**0.18**	-9475	4269	3095	5061	0.73	-1174	1.19	792
ycnJ	probable copper export protein	10942	2043	1997	**0.19**	-8899	**0.18**	-8945	4061	3094	4865	0.76	-967	1.20	804
ycnK	probable transcriptional regulator	15142	3068	2915	**0.20**	**-12074**	**0.19**	**-12226**	5005	2965	5491	0.59	-2040	1.10	485
ydhM	PTS oligo-beta-mannoside-specific enzyme IIB component	3249	1659	2386	0.51	-1590	0.73	-863	8912	1963	1666	0.22	-6949	**0.19**	-7246
ydhN	PTS oligo-beta-mannoside-specific enzyme IIA componen	912	399	619	0.44	-513	0.68	-293	2682	540	437	**0.20**	-2142	**0.16**	-2245
ydhO	PTS oligo-beta-mannoside-specific enzyme IIC component	1419	604	915	0.43	-816	0.64	-504	3905	763	678	**0.20**	-3141	**0.17**	-3227
ydhP	phospho-beta-glucosidase	1252	597	833	0.48	-655	0.66	-420	3756	767	692	**0.20**	-2989	**0.18**	-3063
yfiY	arthrobactin transport system substrate-binding protein, Fur regulon	9444	7734	6422	0.82	-1710	0.68	-3022	16068	4650	8413	0.29	**-11418**	0.52	-7656

Table 7. (continued)

		10 min							30 min						
		Signal Intensity			+End		+Bac		Signal Intensity			+End		+Bac	
Gene	**Product**	**Cont**	**+End**	**+Bac**	**Ratio**	**Dif**	**Ratio**	**Dif**	**Cont**	**+End**	**+Bac**	**Ratio**	**Dif**	**Ratio**	**Dif**
yfmC	ferric citrate transport system substrate-binding protein, Fur regulon	10197	11716	8862	1.15	1519	0.87	-1335	15245	5043	6805	0.33	**-10202**	0.45	-8440
yhfQ	putative iron(III) dicitrate transport system substrate-binding protein	10022	9469	6871	0.94	-553	0.69	-3152	14826	4164	9002	0.28	**-10662**	0.61	-5824
ykbA	probable amino acid permease	2229	897	1108	0.40	-1332	0.50	-1121	2301	466	1109	**0.20**	-1834	0.48	-1192
yutK	probable nucleoside uptake transporter	4828	2318	2678	0.48	-2510	0.55	-2150	4203	784	1490	**0.19**	-3419	0.35	-2713
yvfM	putative maltose/maltodextrin transport system permease protein	419	212	269	0.51	-206	0.64	-150	1337	345	266	0.26	-992	**0.20**	-1071
yxeB	ferrioxamine transport system substrate-binding protein, Fur regulon	8355	10163	5269	1.22	1808	0.63	-3086	14436	3870	8528	0.27	**-10567**	0.59	-5909
(Cell membrane associated protein coding genes and operons; 14 genes)															
atpA	ATP synthase alpha chain	25993	14177	15037	0.55	**-11815**	0.58	**-10956**	21488	7788	15037	0.36	**-13699**	0.70	-6451
atpB	ATP synthase a chain	24071	12447	13383	0.52	**-11624**	0.56	**-10688**	20272	6417	13173	0.32	**-13855**	0.65	-7099
atpC	ATP synthase epsilon chain	22974	12426	13253	0.54	**-10548**	0.58	-9721	20143	7139	13253	0.35	**-13004**	0.66	-6891
atpD	ATP synthase beta chain	26348	15134	15711	0.57	**-11214**	0.60	**-10637**	22315	8815	16209	0.40	**-13500**	0.73	-6106
atpE	ATP synthase c chain	24846	12828	14009	0.52	**-12018**	0.56	**-10837**	21688	7259	14618	0.33	**-14429**	0.67	-7070
atpF	ATP synthase b chain	28034	17137	18538	0.61	**-10896**	0.66	-9495	27067	11225	19493	0.41	**-15842**	0.72	-7574
atpG	ATP synthase gamma chain	27374	16318	17179	0.60	**-11056**	0.63	**-10194**	24128	9885	17974	0.41	**-14243**	0.74	-6154
atpH	ATP synthase delta chain	26993	15334	17063	0.57	**-11659**	0.63	-9930	23750	9000	17063	0.38	**-14750**	0.72	-6687
ctaB	cytochrome caa3 oxidase assembly factor	13868	3889	3681	0.28	-9980	0.27	**-10188**	9374	3101	6514	0.33	-6273	0.69	-2860

Table 7. (continued)

		10 min							30 min						
		Signal Intensity			+End		+Bac		Signal Intensity			+End		+Bac	
Gene	Product	Cont	+End	+Bac	Ratio	Dif	Ratio	Dif	Cont	+End	+Bac	Ratio	Dif	Ratio	Dif
qoxA	cytochrome aa3 quinol oxidase subunit II	25109	11865	12538	0.47	**-13244**	0.50	**-12571**	21756	6646	13293	0.31	**-15110**	0.61	-8463
qoxB	cytochrome aa3 quinol oxidase subunit I	24415	12302	12931	0.50	**-12113**	0.53	**-11484**	20275	6714	12931	0.33	**-13561**	0.64	-7344
qoxC	cytochrome aa3 quinol oxidase subunit III	29442	17702	18314	0.60	**-11740**	0.62	**-11128**	26634	10329	18398	0.39	**-16305**	0.69	-8236
yhcK	conserved membrane protein	3139	1750	1867	0.56	-1389	0.59	-1273	3221	482	1368	**0.15**	-2739	0.42	-1853
yxaI	conserved membrane protein	2331	833	988	0.36	-1498	0.42	-1343	2974	352	372	**0.12**	-2621	**0.13**	-2601
(Translation machinery genes; 8 genes)															
rplJ	50S ribosomal protein L10	34429	32304	32617	0.94	-2125	0.95	-1812	32980	20594	30669	0.62	**-12386**	0.93	-2312
rpmE	50S ribosomal protein L31	28677	24634	26050	0.86	-4043	0.91	-2627	25587	13216	21389	0.52	**-12371**	0.84	-4199
rpmI	50S ribosomal protein L35	30748	29854	30101	0.97	-894	0.98	-647	30746	19444	27315	0.63	**-11303**	0.89	-3431
rpsI	30S ribosomal protein S9	28723	27942	26846	0.97	-781	0.93	-1877	27507	15608	25351	0.57	**-11899**	0.92	-2156
rpsJ	30S ribosomal protein S10	37920	37767	36363	1.00	-153	0.96	-1557	35398	24460	34162	0.69	**-10938**	0.97	-1236
tsf	elongation factor Ts	28672	27470	27685	0.96	-1202	0.97	-987	28856	18795	25125	0.65	**-10062**	0.87	-3731
ybxF	ribosomal protein L7AE family	37462	35992	35851	0.96	-1470	0.96	-1612	34517	21373	31620	0.62	**-13144**	0.92	-2897
infC	translation initiation factor IF-3	28364	27407	27744	0.97	-957	0.98	-620	26214	16163	24306	0.62	**-10052**	0.93	-1908
(Mtabolic enzymecoding gene and operon; 11 genes)															
dacA	D-alanyl-D-alanine carboxypeptidase	17276	11510	13089	0.67	-5766	0.76	-4188	17413	6530	14412	0.37	**-10884**	0.83	-3002
lipB	extracellular lipase	1524	680	974	0.45	-844	0.64	-549	2803	504	828	**0.18**	-2299	0.30	-1975
manA	mannose-6-phosphate isomerase	27756	17246	19994	0.62	**-10510**	0.72	-7763	23281	28158	30392	1.21	4877	1.31	7111
pyrG	CTP synthase	15881	14273	13843	0.90	-1608	0.87	-2038	16803	5851	9246	0.35	**-10952**	0.55	-7557
pyrH	uridylate kinase	20935	16701	16966	0.80	-4233	0.81	-3969	20884	9905	16966	0.47	**-10979**	0.81	-3918
xynB	xylan beta-1,4-xylosidase	157	151	174	0.96	-6	1.11	17	822	172	165	0.21	-650	**0.20**	-657

Table 7. (continued)

		10 min							30 min						
		Signal Intensity			+End		+Bac		Signal Intensity			+End		+Bac	
Gene	Product	Cont	+End	+Bac	Ratio	Dif	Ratio	Dif	Cont	+End	+Bac	Ratio	Dif	Ratio	Dif
yaaD	synthase subunit of a glutamine amidotransferase essential for B(6) biosynthesis	10693	5441	6270	0.51	-5253	0.59	-4423	15632	5443	9508	0.35	**-10189**	0.61	-6124
ydhS	mannose-6-phosphate isomerase	1001	470	634	0.47	-531	0.63	-367	3857	1028	722	0.27	-2829	**0.19**	-3135
yjlC	function unknown and unique	19064	15209	15669	0.80	-3855	0.82	-3395	20472	8328	13345	0.41	**-12144**	0.65	-7127
yjlD	similar to NADH dehydrogenase, FAD-containing subunit	17902	16076	16119	0.90	-1826	0.90	-1783	21752	9842	15121	0.45	**-11911**	0.70	-6631
yutJ	similar to NADH dehydrogenase	5443	3168	3515	0.58	-2275	0.65	-1929	9256	1686	3727	**0.18**	-7570	0.40	-5529
(Others; 13 genes)															
bdbA	thiol-disulfide oxidoreductase, SP-beta protein	938	184	281	**0.20**	-754	0.30	-657	3898	263	1022	**0.07**	-3636	0.26	-2876
yolJ	similar to glycosyltransferase, SP-beta protein	1095	375	483	0.34	-720	0.44	-611	4538	431	1398	**0.10**	-4107	0.31	-3140
bdbB	disulfide bond formation protein, SP-beta protein	779	282	355	0.36	-497	0.46	-423	2800	320	917	**0.11**	-2480	0.33	-1883
cspB	major cold-shock protein	31828	28892	28784	0.91	-2937	0.90	-3044	31727	21625	29914	0.68	**-10102**	0.94	-1813
fnr	transcriptional regulator of anaerobic genes	2724	610	533	0.22	-2114	**0.20**	-2191	1117	750	1137	0.67	-368	1.02	20
gltT	transcriptional activator of the glutamate synthase operon	13359	9053	10256	0.68	-4306	0.77	-3103	18292	6639	12500	0.36	**-11653**	0.68	-5792
wapA	cell wall-associated protein	21046	20295	21889	0.96	-751	1.04	843	18918	4226	6439	0.22	**-14691**	0.34	**-12479**
wprA	cell-wall-associated protease precursor	13818	6842	8827	0.50	-6977	0.64	-4991	19429	6907	13170	0.36	**-12521**	0.68	-6259
yxxG	function unknown and unique	21106	17279	20653	0.82	-3827	0.98	-453	20278	4900	7080	0.24	**-15378**	0.35	**-13198**
yfmQ	function unknown and unique	9795	1738	1607	**0.18**	-8056	**0.16**	-8188	4285	2269	3557	0.53	-2017	0.83	-729
ypjA	conserved protein	1063	639	856	0.60	-424	0.81	-206	1057	216	679	**0.20**	-841	0.64	-378
yufK	function unknown and unique	3477	1153	1553	0.33	-2323	0.45	-1924	3492	606	1407	**0.17**	-2886	0.40	-2085
yvbI	function unknown and unique	2978	855	1070	0.29	-2122	0.36	-1908	5401	1006	1296	**0.19**	-4396	0.24	-4105

IV. CONCLUSIONS

The responses of bacterial cells to challenge by external antibiotics are very complicated, consisting of relatively specific responses to the dysfunctional activities of antibiotics and rather general responses to ensure cell functionality. The robustness of the latter responses often makes it difficult to evaluate the contribution of any particular genetic system, because the inactivation of one system might be compensated for by induction of other systems. However, an understanding of bacterial cell responses to antibiotics, at the molecular level, is important to understand the mechanisms of action of antibiotics and the emergency responses of antibiotic resistance systems. Toward this goal, transcriptional responses of *B. subtilis* cells to antibiotics have been extensively studied as a model system.

In the present work, we used a high-density tiling chip to assess transcriptional responses in *B. subtilis* cells challenged by antibiotics. Using the quantitative advantage of the tiling chip, we introduced a new criterion, an increase in transcriptional level, in addition to the conventional induction ratio, to identify genes of biological significance among genes with lower induction ratios. Our results indicate that introduction of this criterion led to unambiguous identification of core transcription responses to antibiotics, with a reduction in the number of possible background genes, compared to previous results employing gene arrays. Indeed, induction of about half of the genes belonging to the SigM and Spx regulons was identified, using induction levels to distinguish these genes from those of numerous genes with lower induction ratios. Although the minimum induction level to identify significantly upregulated genes was arbitrarily set at 10,000 units, it is interesting to note that among 35 genes added to the upregulated gene list by decreasing the criterion

to 8,000 units, 14 belonged to the SigM or Spx regulons (data not shown), indicating that resetting of the minimum value would not greatly affect the main conclusions of our work.

In addition, time course analysis showed potential to clarify the complex and dynamic changes in the transcriptome caused by the need to adapt to antibiotic stress. Timecourse analysis suggested that expression of several genes might be transiently induced by initial perturbations in cell envelope structure. However, induction of transcription of genes that are more directly involved in antibiotic resistance persists for longer periods.

The present study, which represents the first transcriptomic analysis of responses of a bacterial cell to challenge by enduracidin, showed that multiple factors contribute to enduracidin resistance. Notably, we determined that inactivation of LiaRS TCS resulted in increased sensitivity to enduracidin, probably caused by a failure to induce LiaHI proteins. Enduracidin may have the ability to cause a distortion of membrane structure similar to that created by the related antibiotic daptomycin, which needs to be counteracted by LiaH to ensure cell functionality. We also demonstrated that LiaI, conserved in *Bacillus* species, plays an important role in supporting LiaH function. These findings contribute to a further understanding of the molecular mechanisms of action of enduracidin and daptomycin, and the role of LiaHI in maintaining the integrity of membrane structure. Consistent with induction of the SigM regulon, inactivation of *sigM* increased sensitivity to enduracidin, to a level similar to that of the *liaR* mutant. In addition, and unexpectedly, SigX also seemed to contribute to enduracidin resistance. Further studies are necessary to clarify the molecular mechanisms of involvement of these sigma factors in enduracidin resistance. In

addition, and for the first time, we found that the Spx regulon was induced in cells challenged by antibiotics, suggesting that thiol-oxidative stress would develop in cells treated with enduracidin or bacitracin. Upregulation of *spx* expression by SigM may contribute to Spx regulon induction. However, the biological significance of this induction in the context of antibiotic resistance awaits further examination, as *spx* inactivation did not affect drug sensitivity.

We used a custom tiling chip to quantitatively monitor the genome-wide transcriptional profile. It is now possible to analyze transcriptional profiles quantitatively by massive sequencing of cDNAs using new high-throughput sequencers (Wang et al., 2009), instead of employing a high-density tiling array. Thus, quantitative transcriptome analysis is now available for any bacterium the genome sequence of which is known, and may be employed to more clearly disclose dynamic transcriptional responses of bacteria to environmental stimuli, including challenge by an antibiotic.

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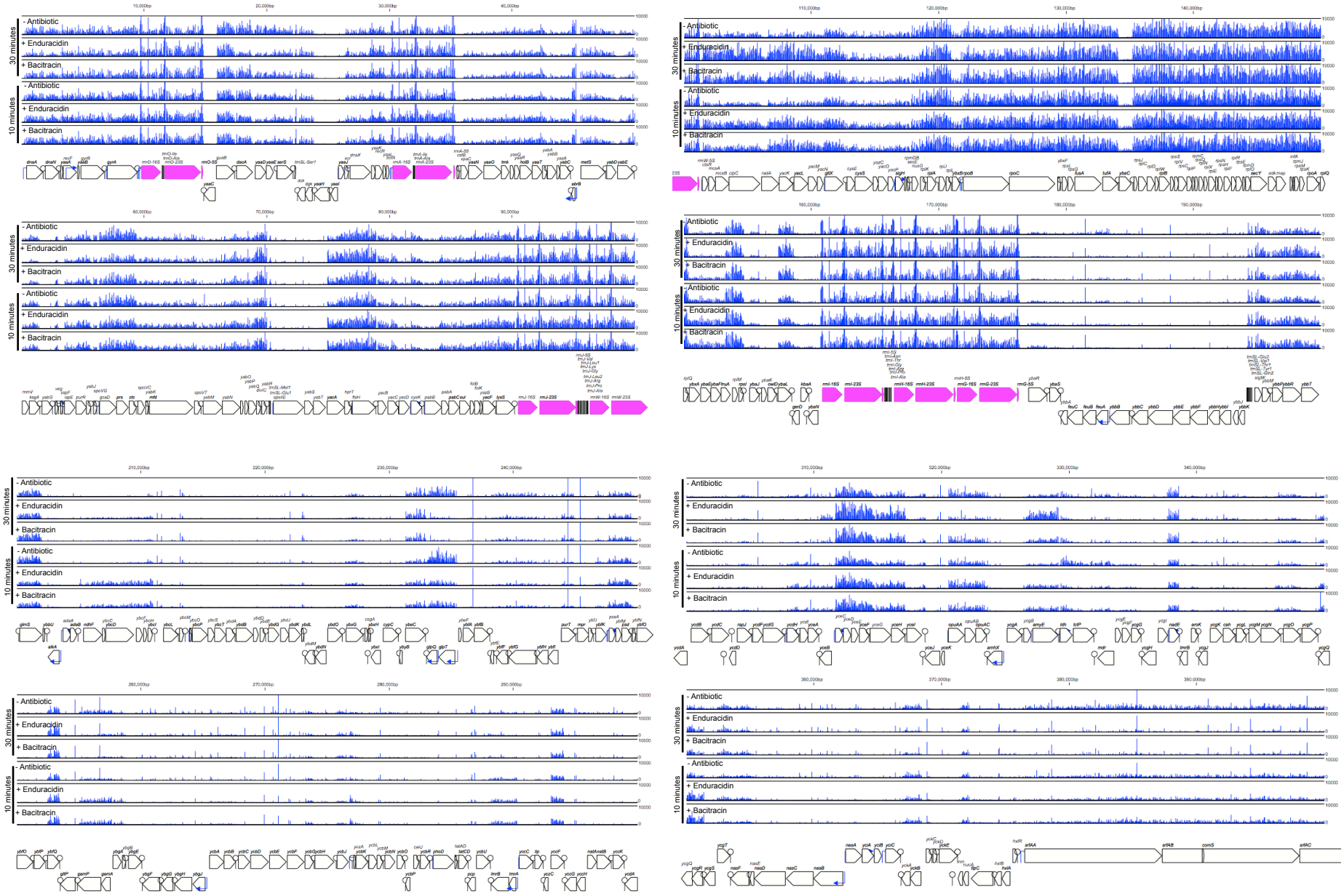
Supplementary Figure 1. Transcriptome profiles on the whole *Bacillus. subtilis* genome



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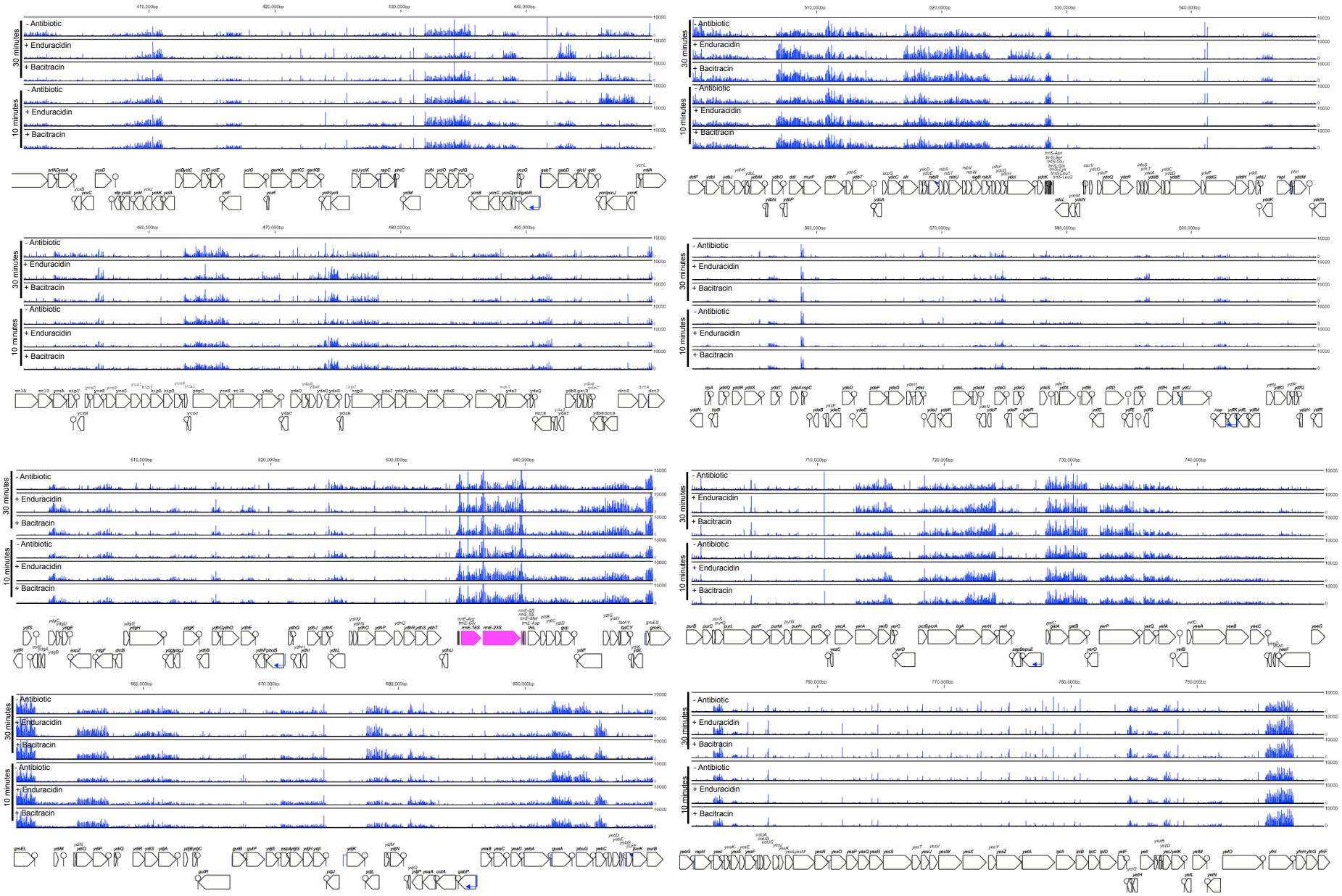

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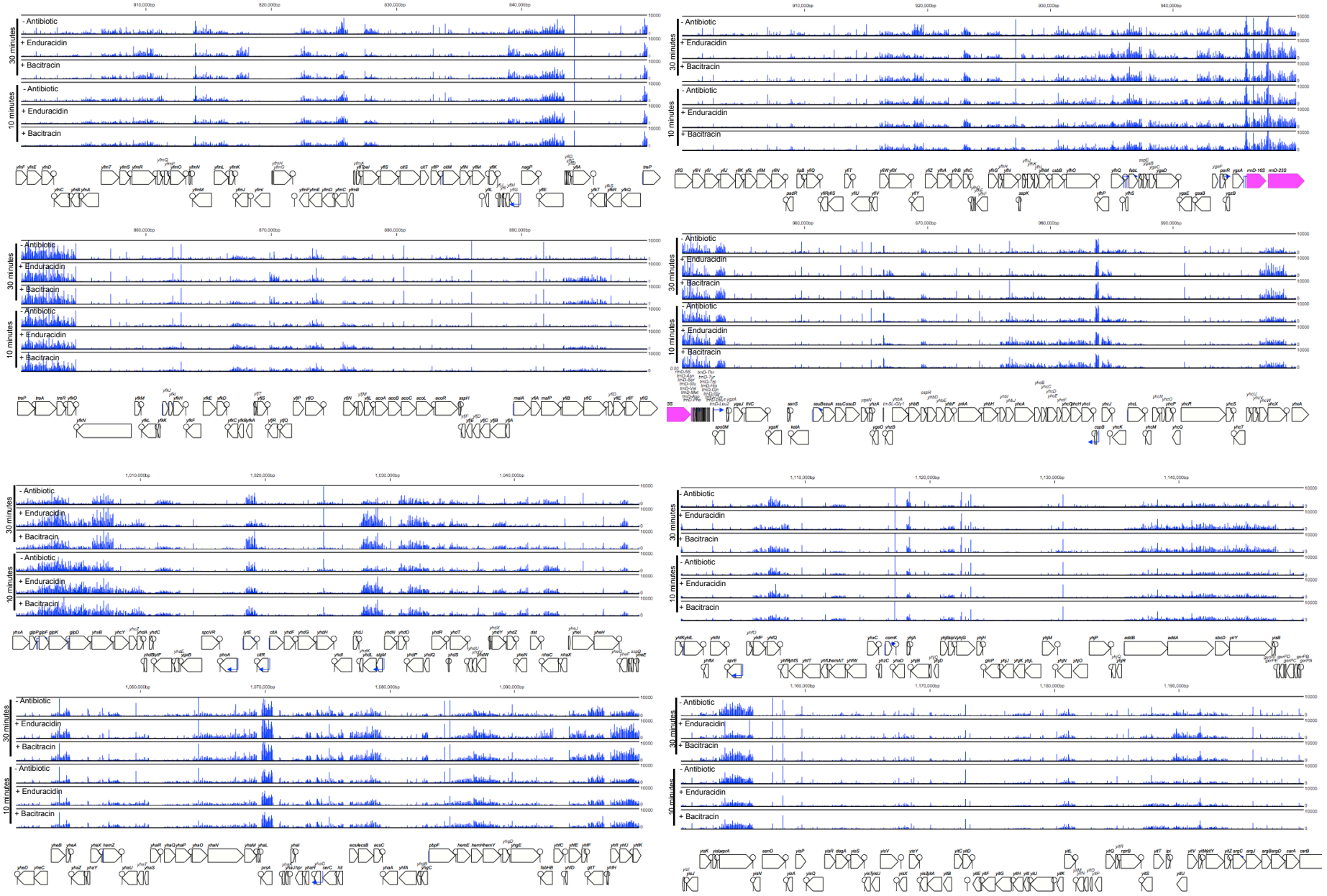

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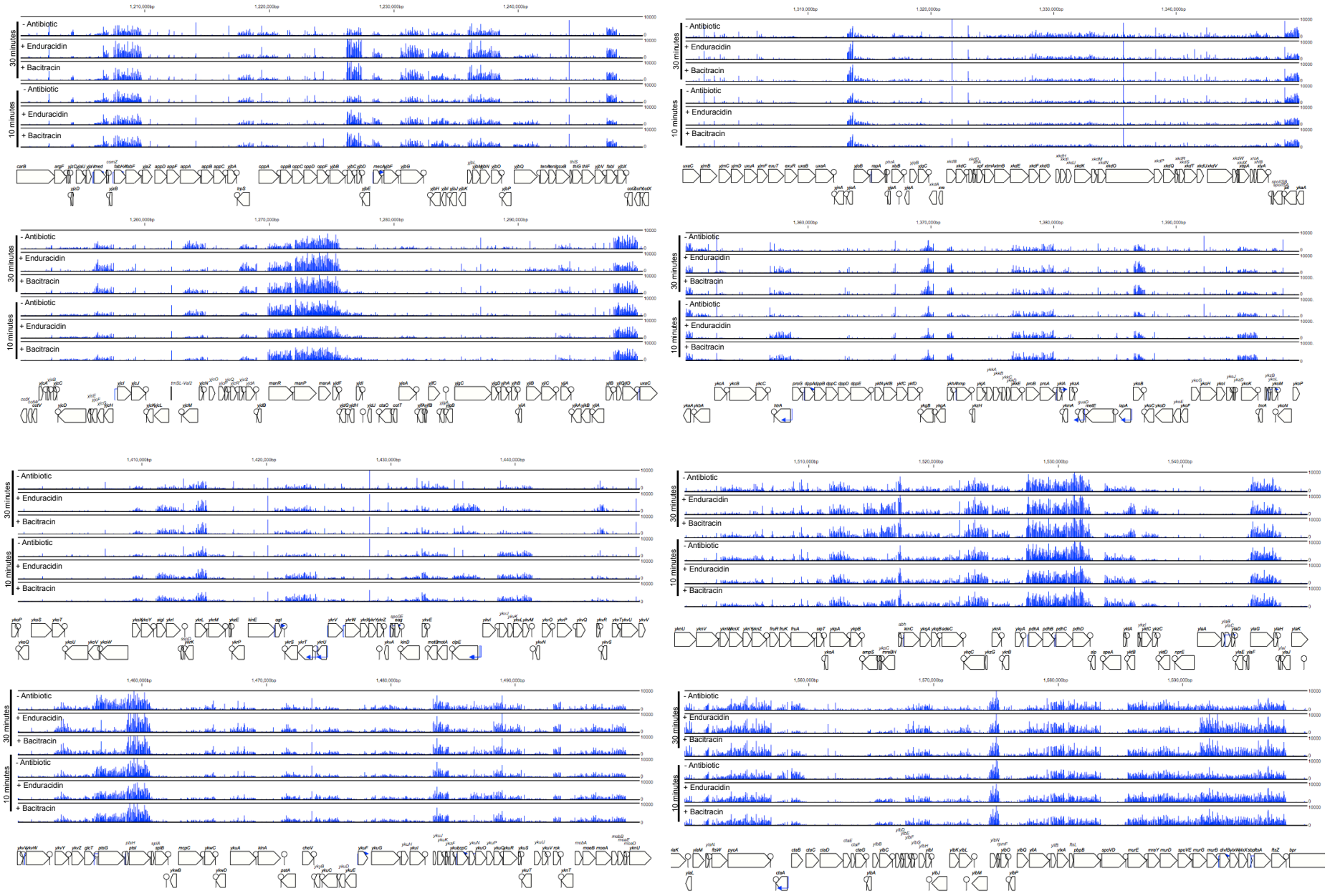

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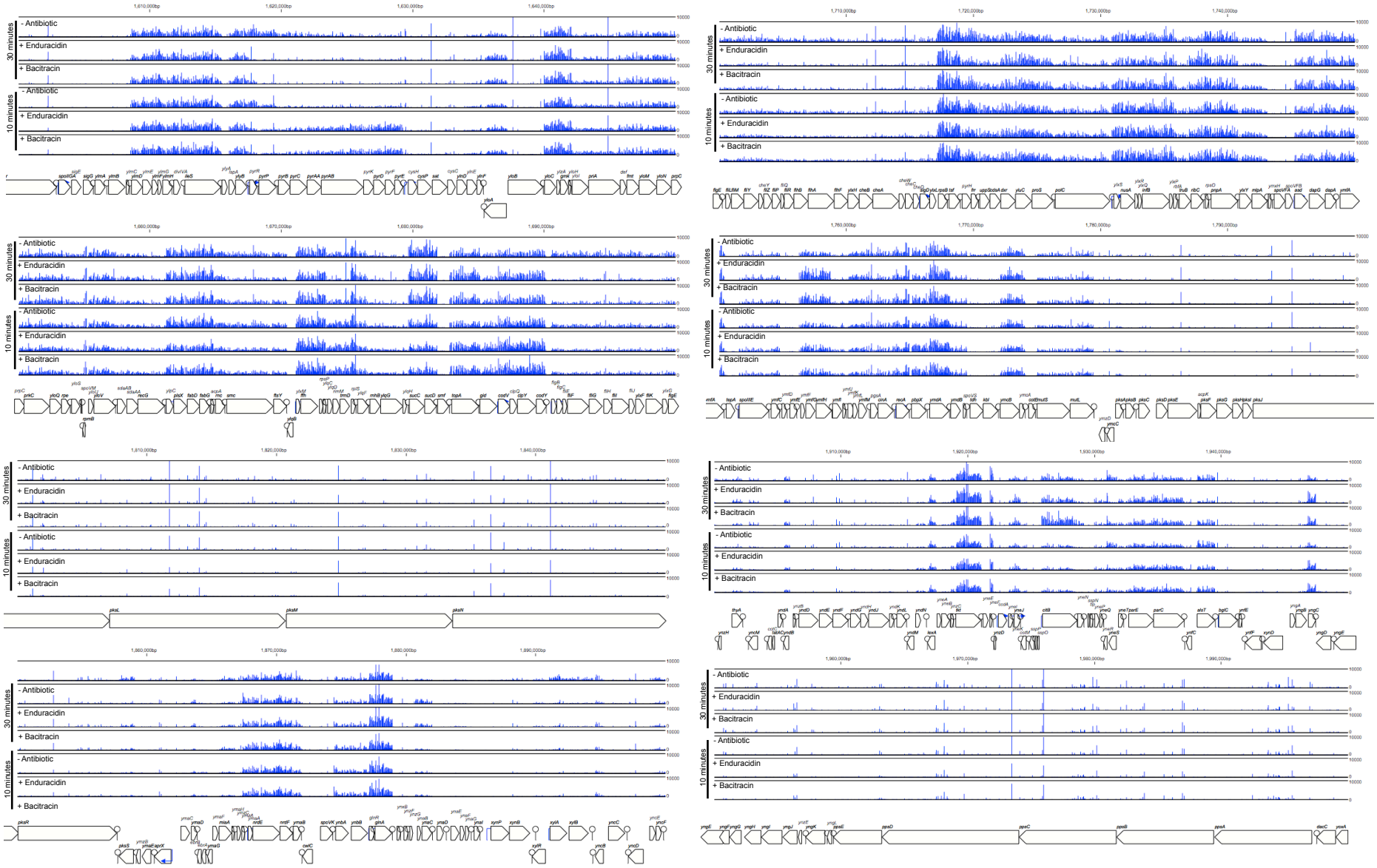

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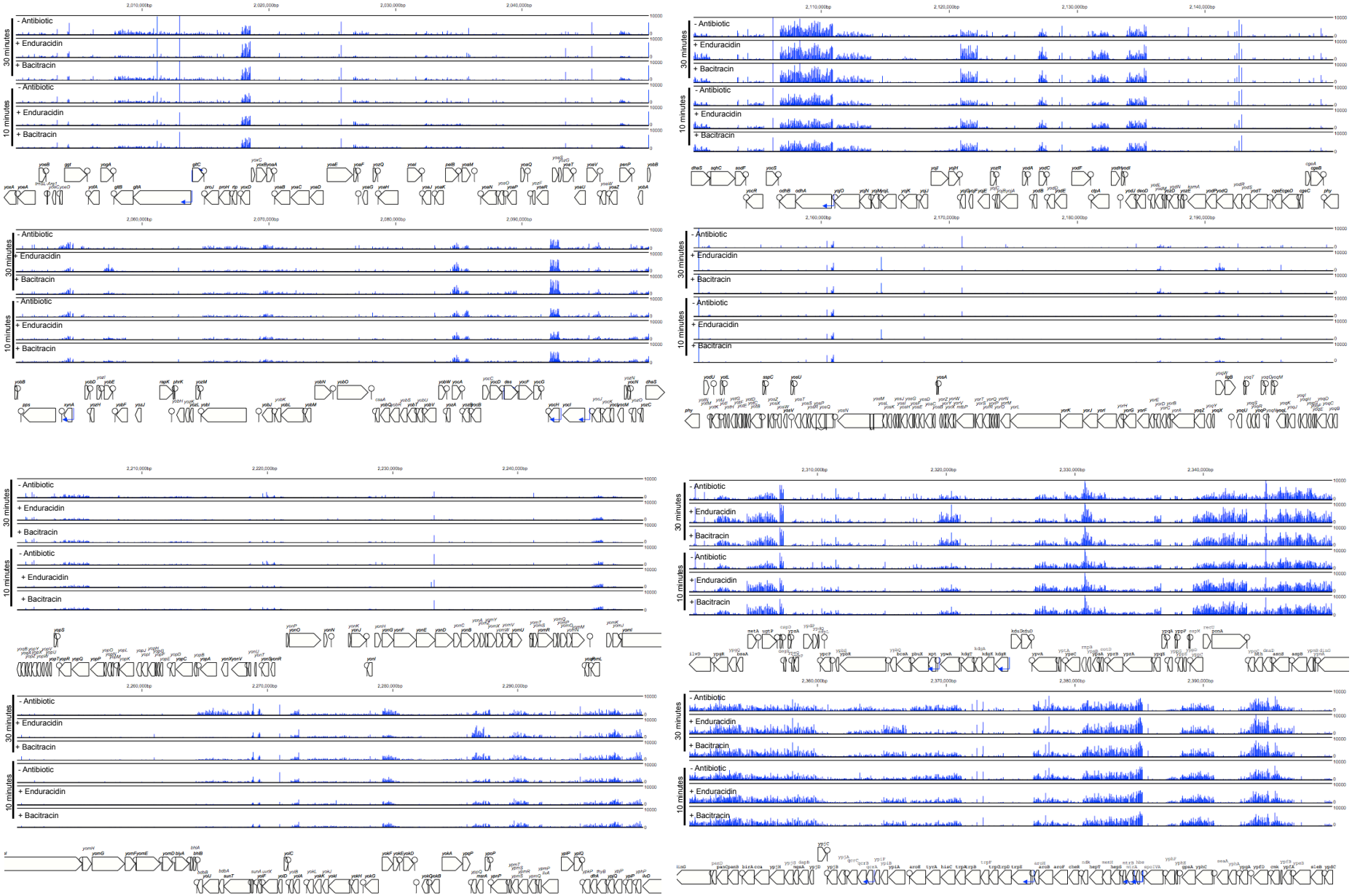

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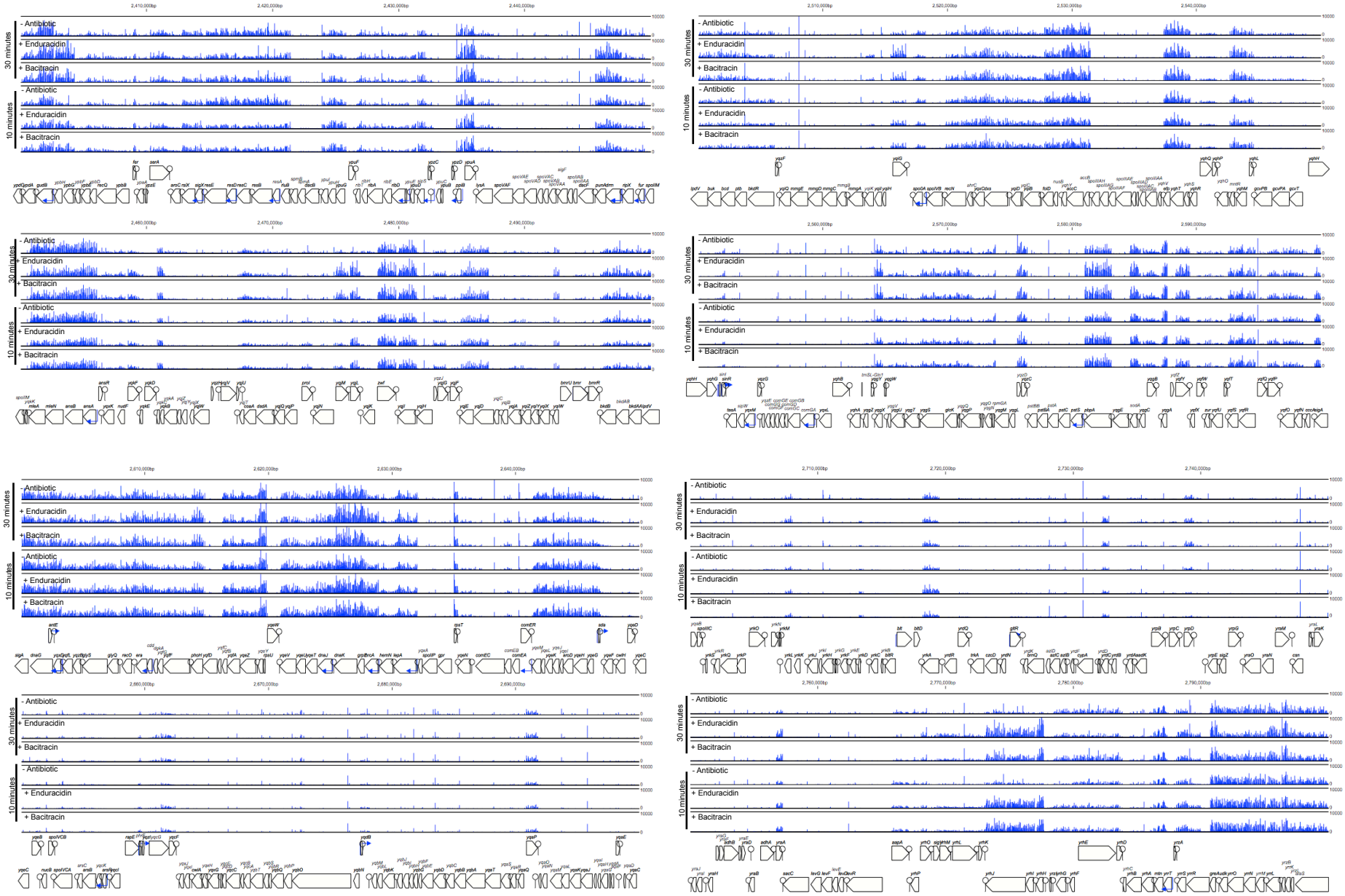

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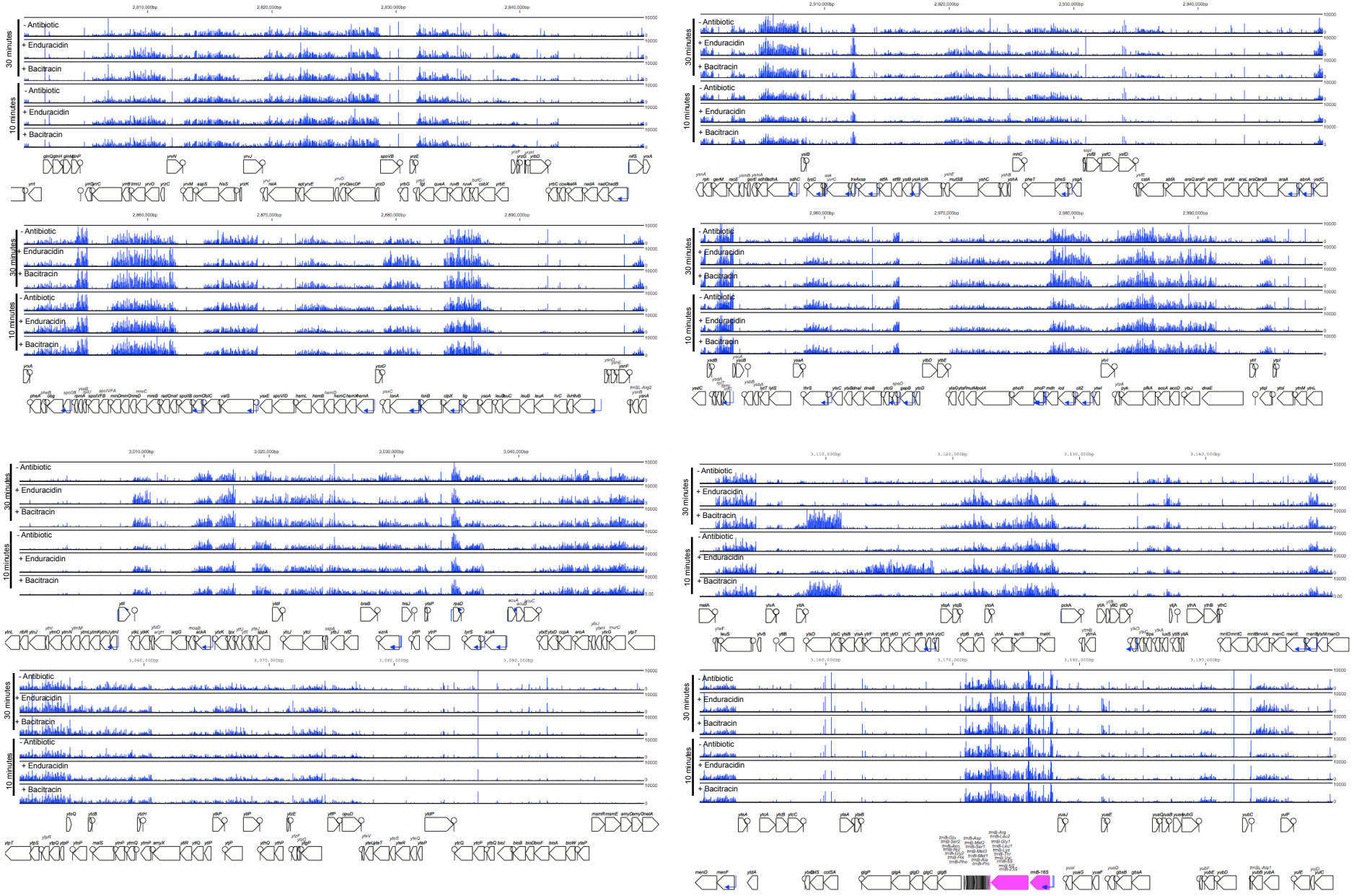

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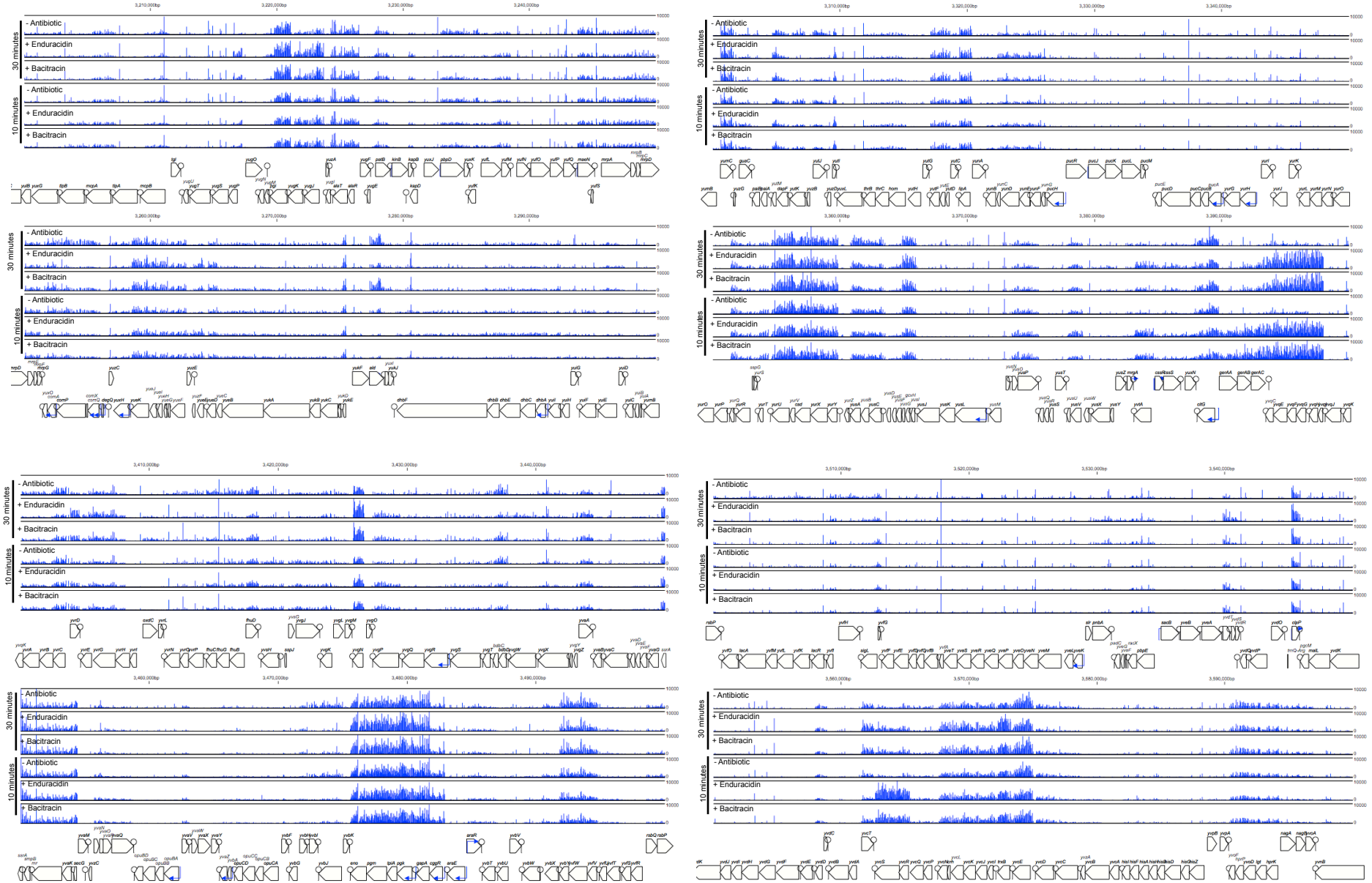

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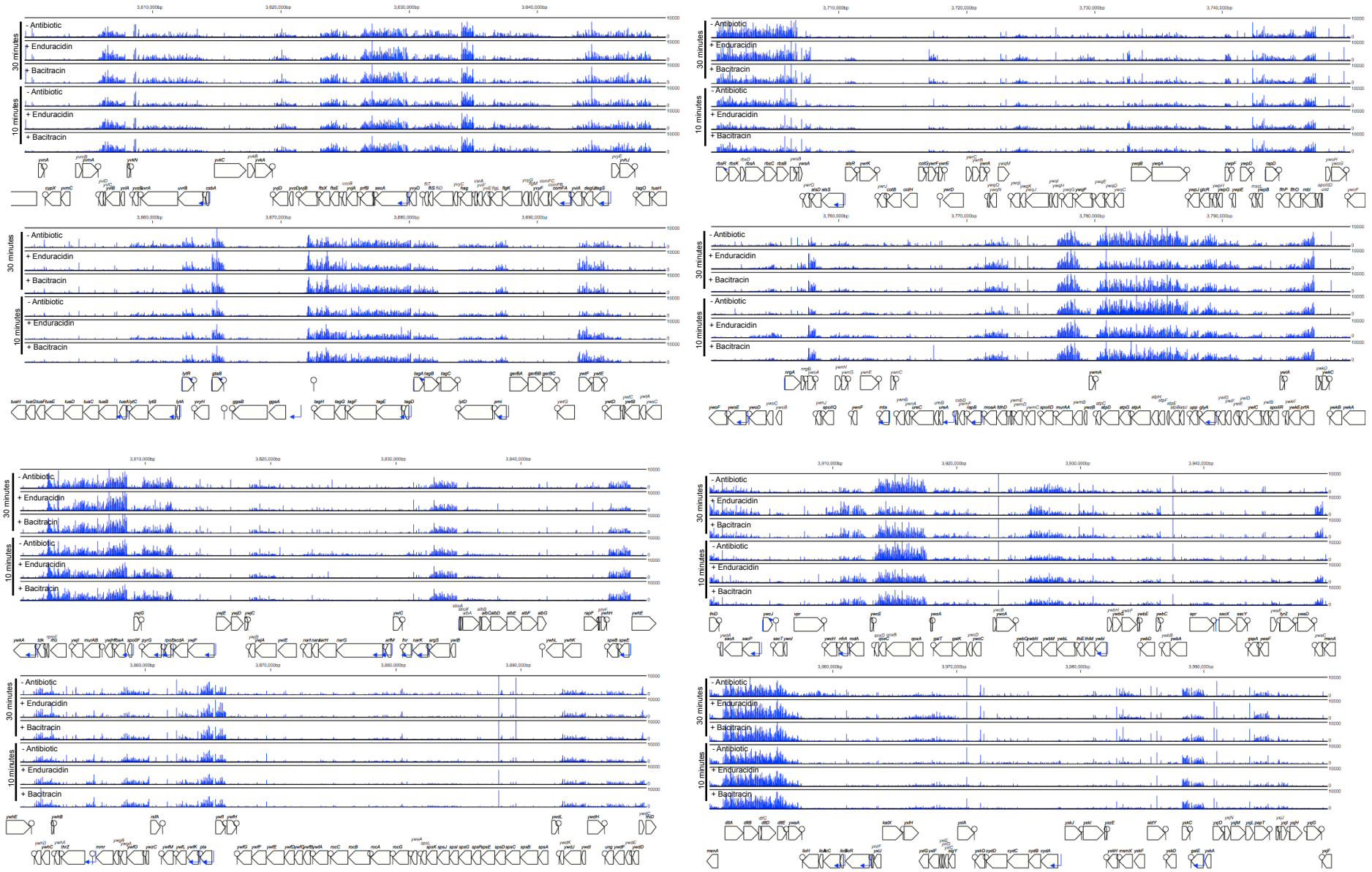

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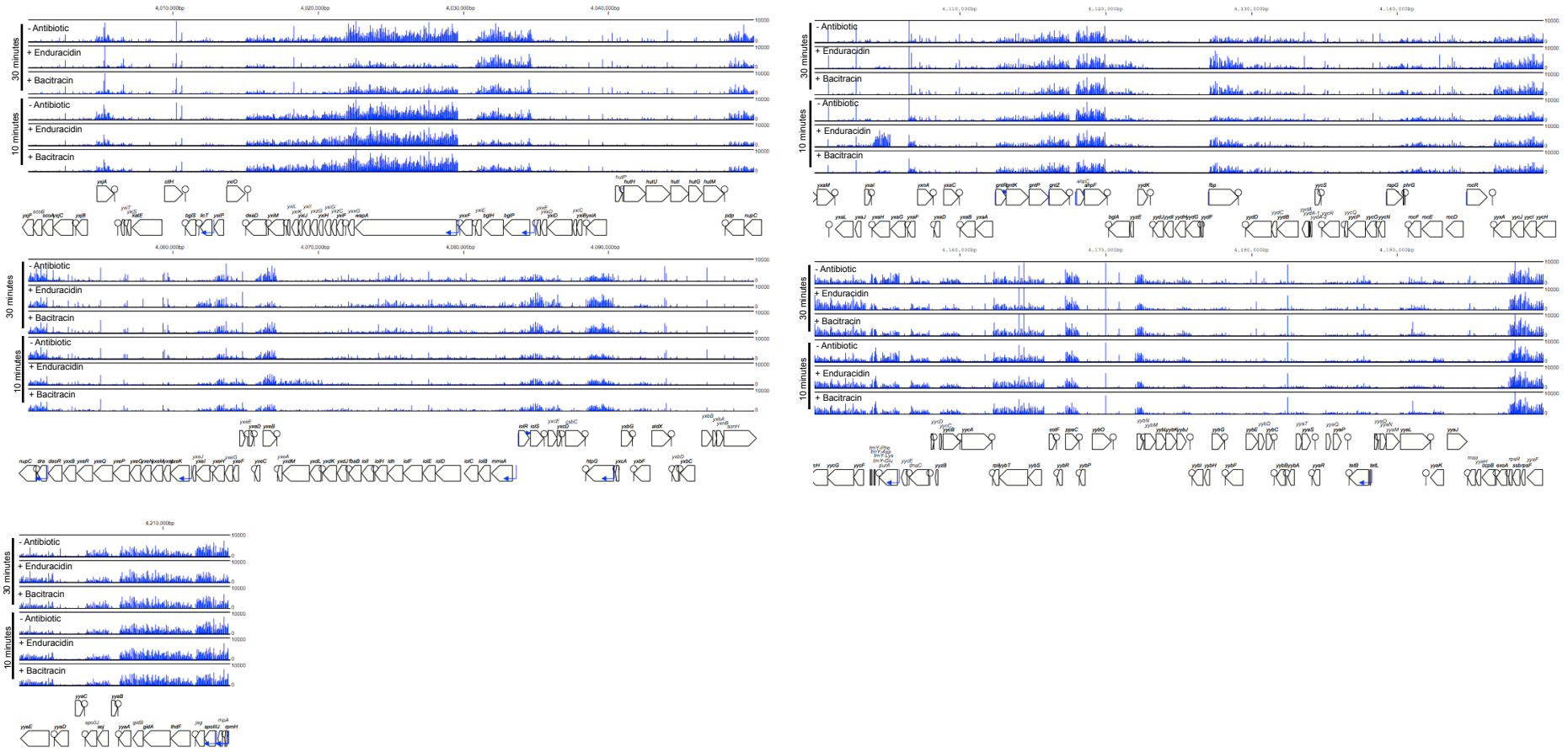
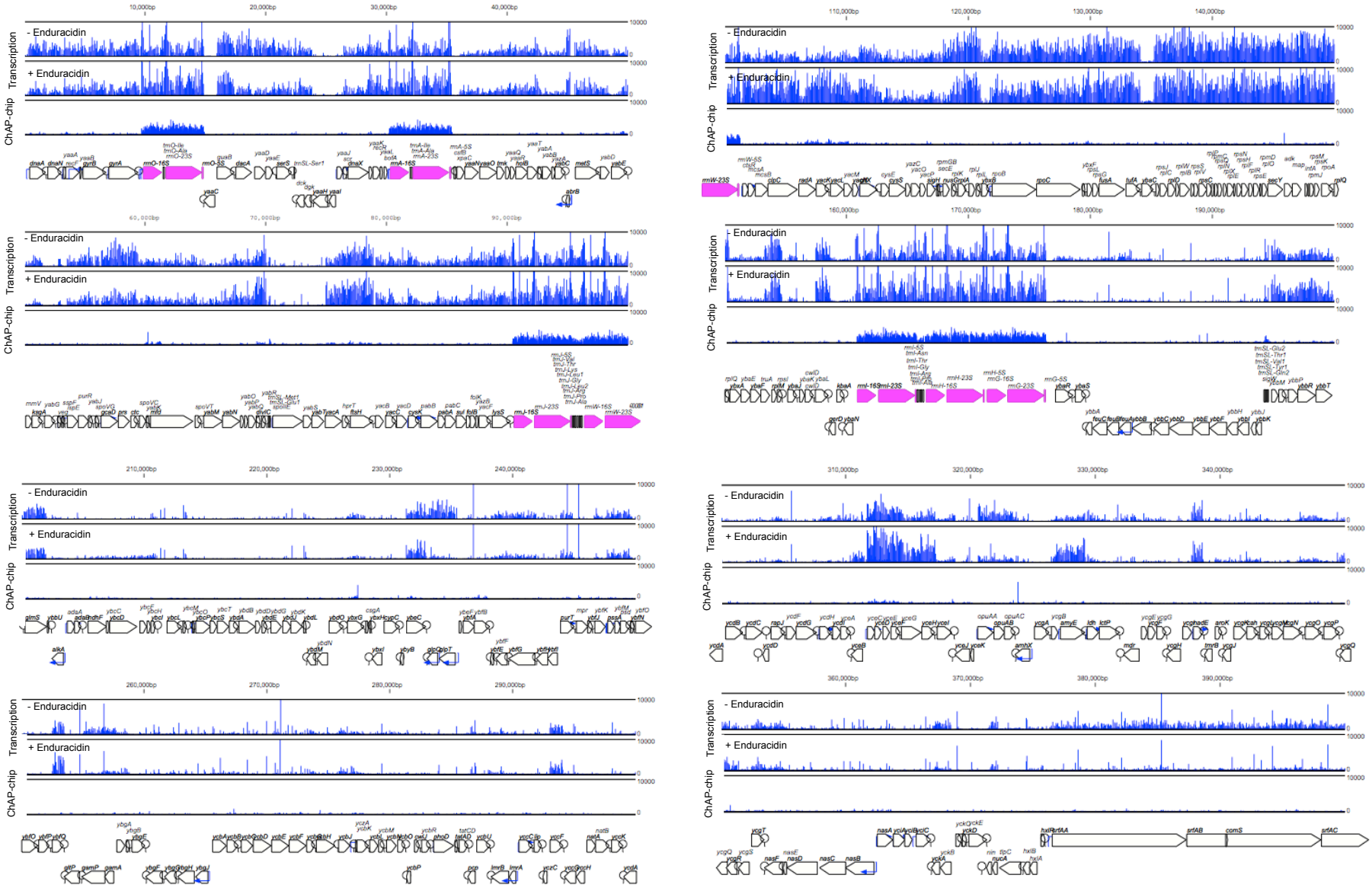


Supplementary Figure 2. Distribution of LiaR binding signals on the whole *Bacillus subtilis* genome



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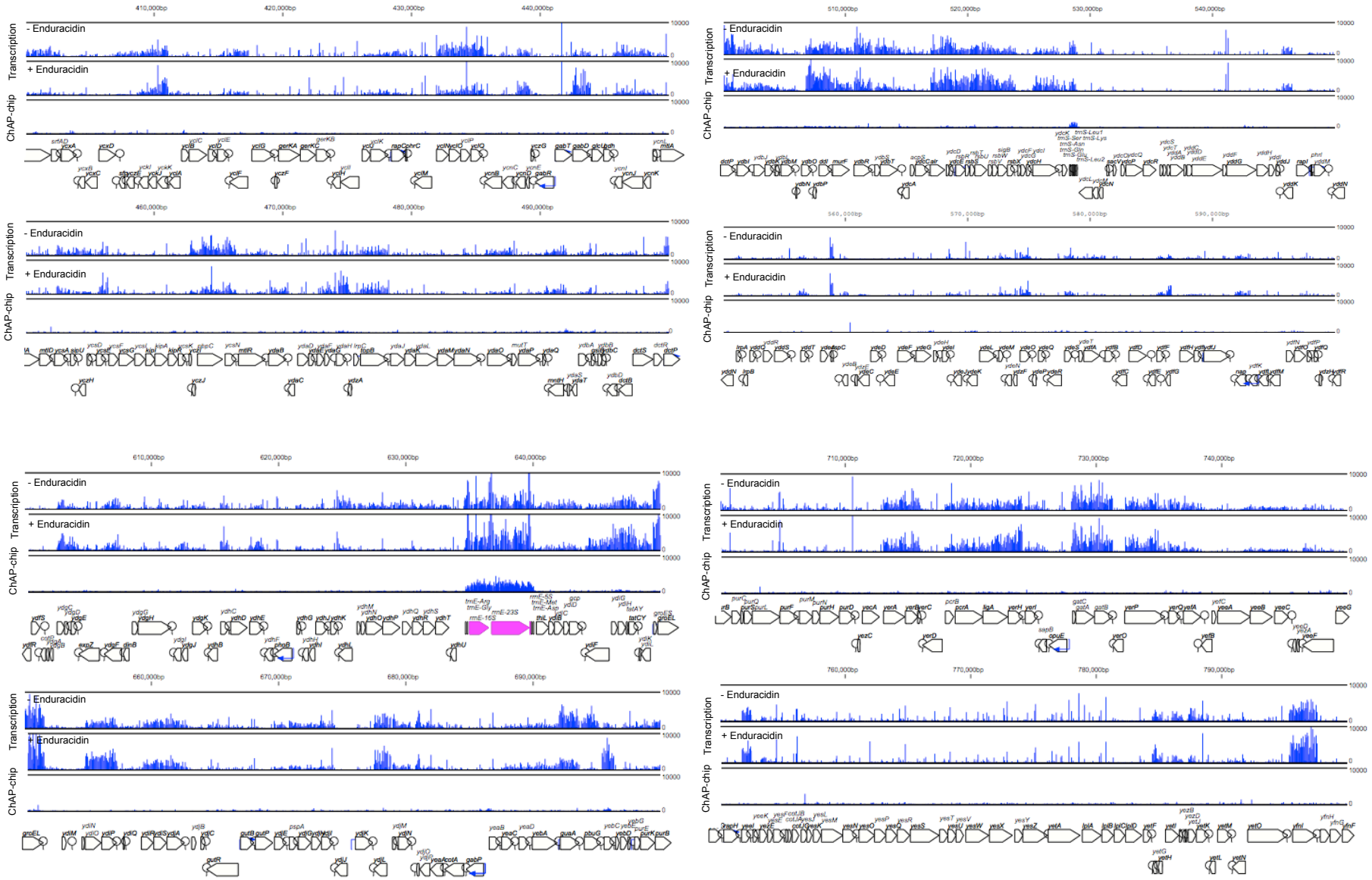

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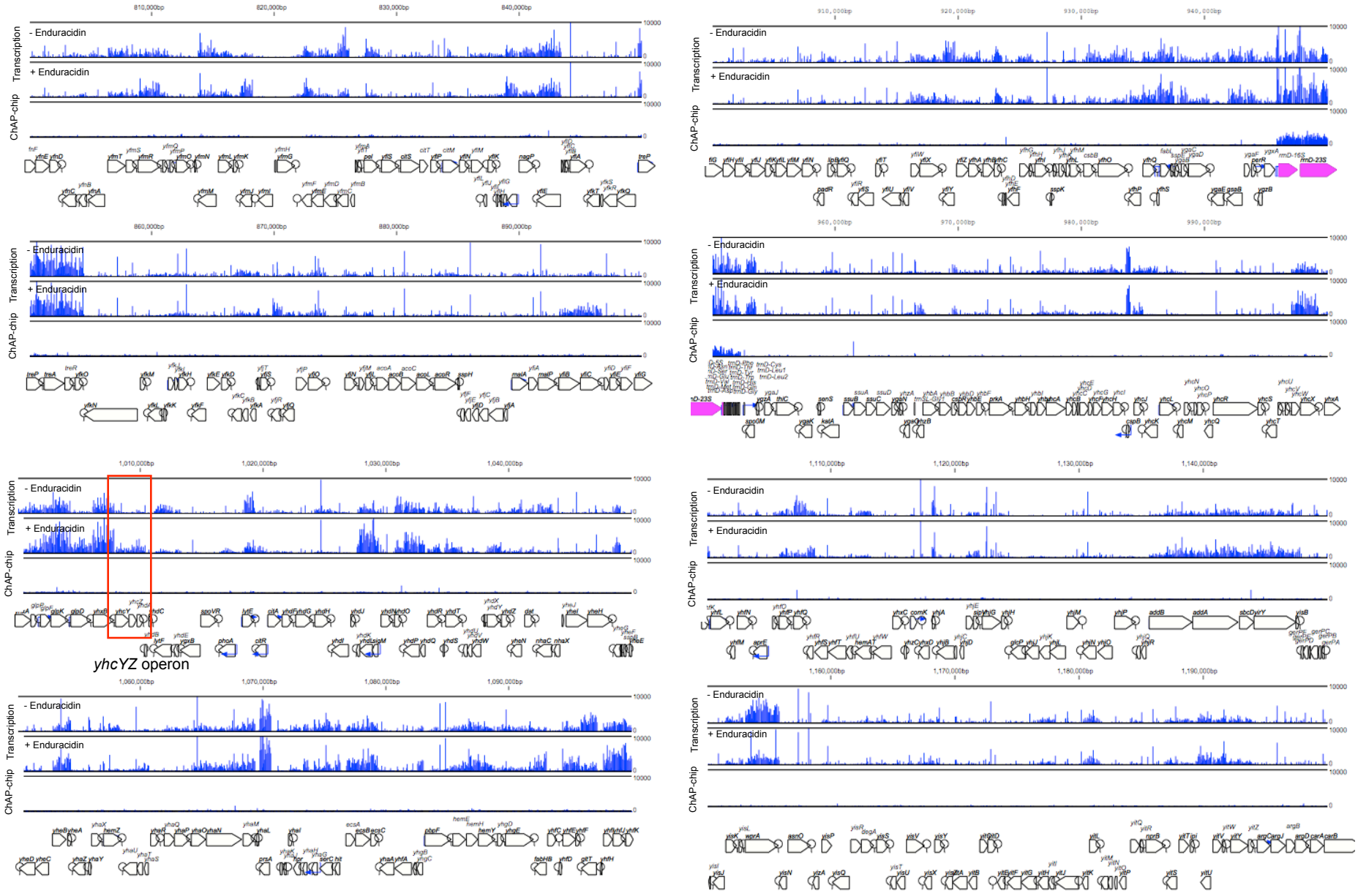

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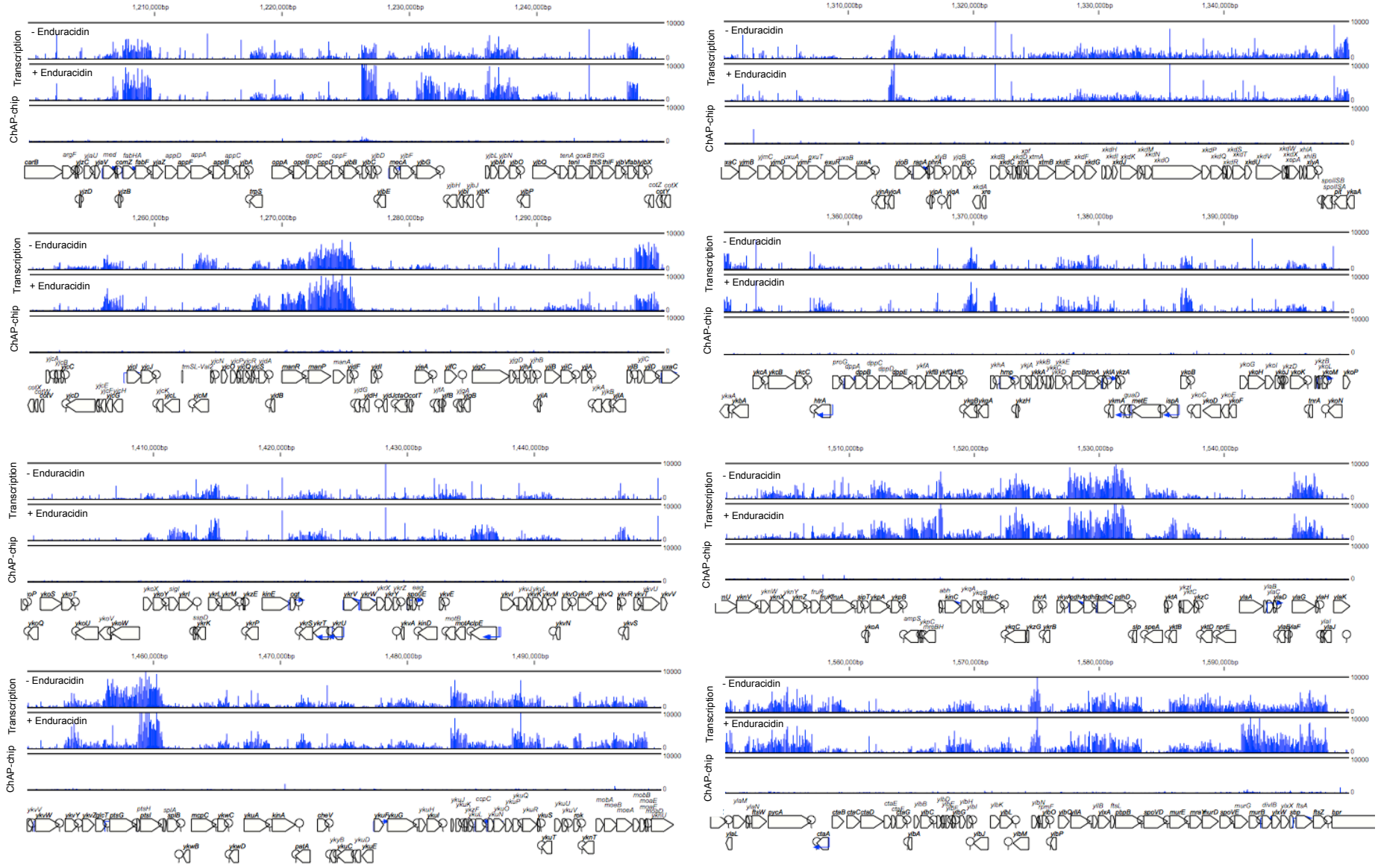

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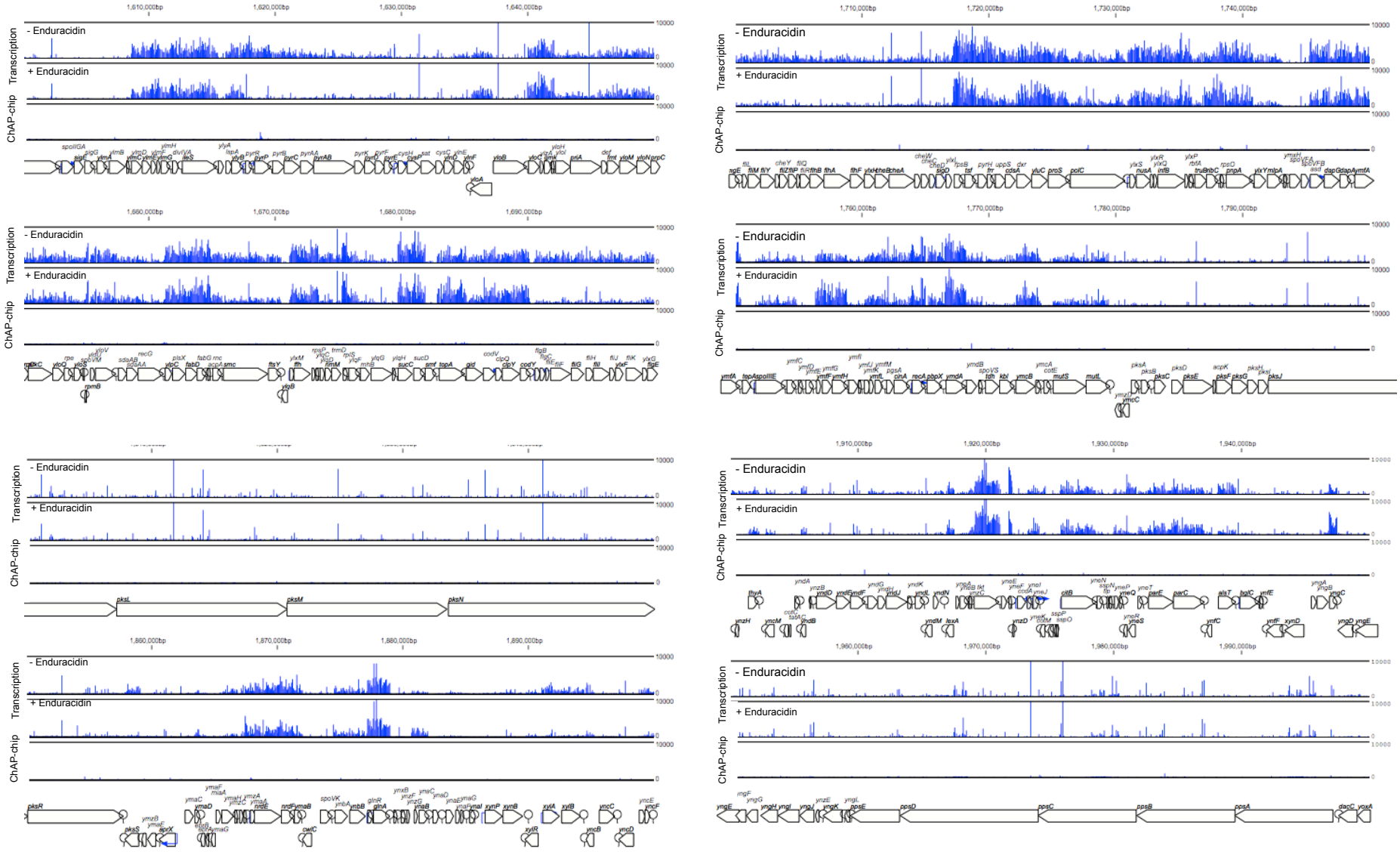

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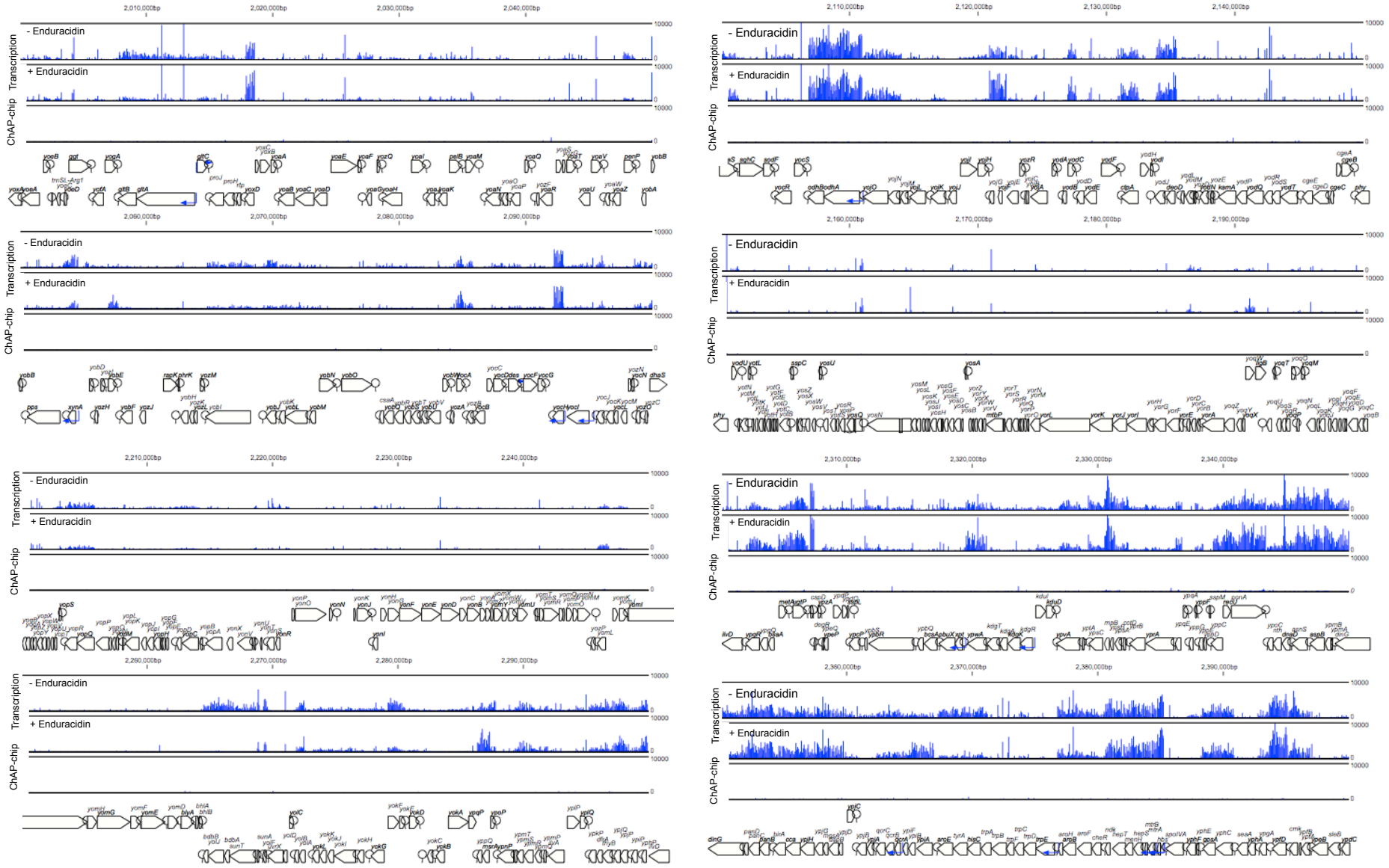

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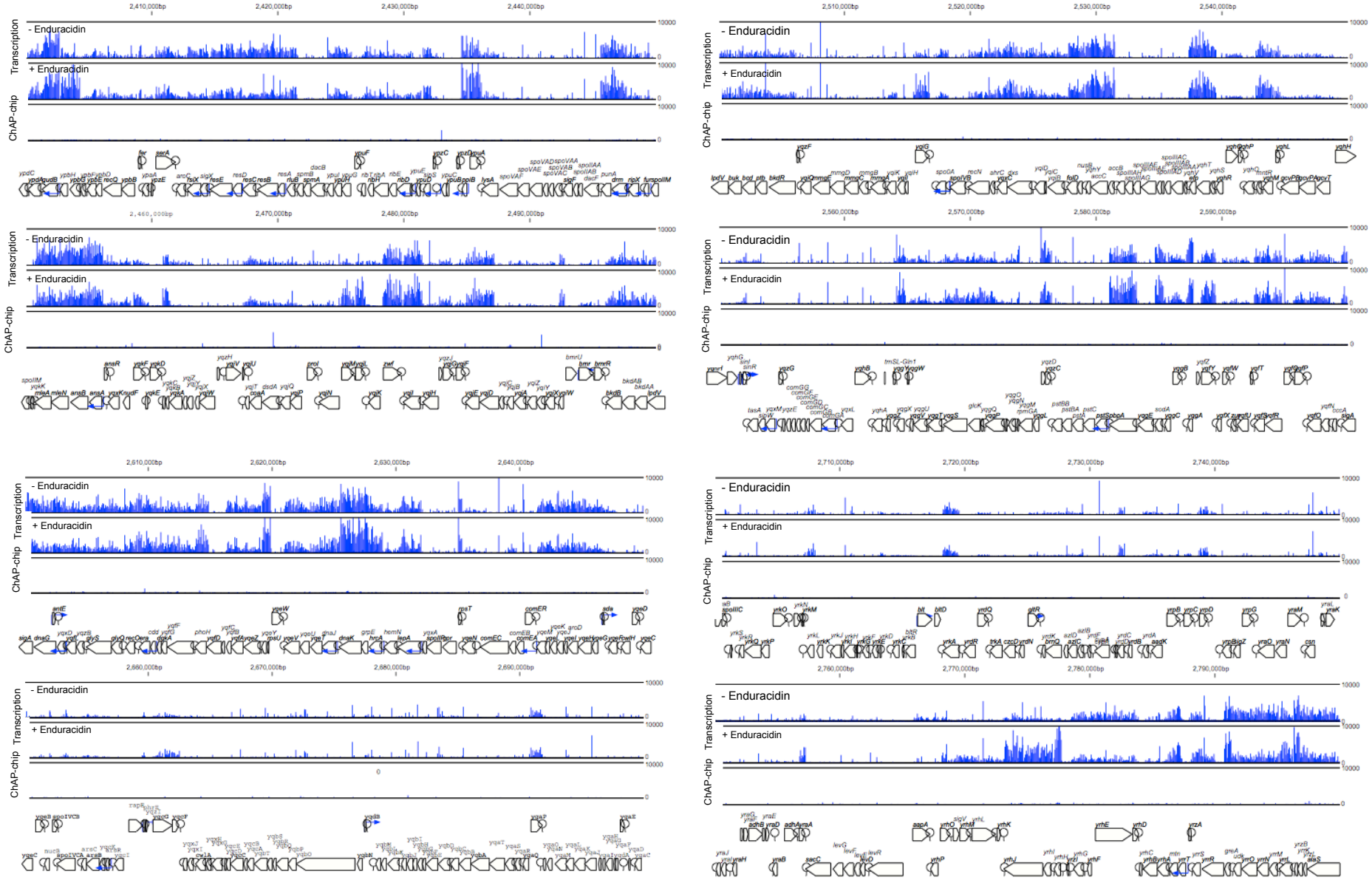

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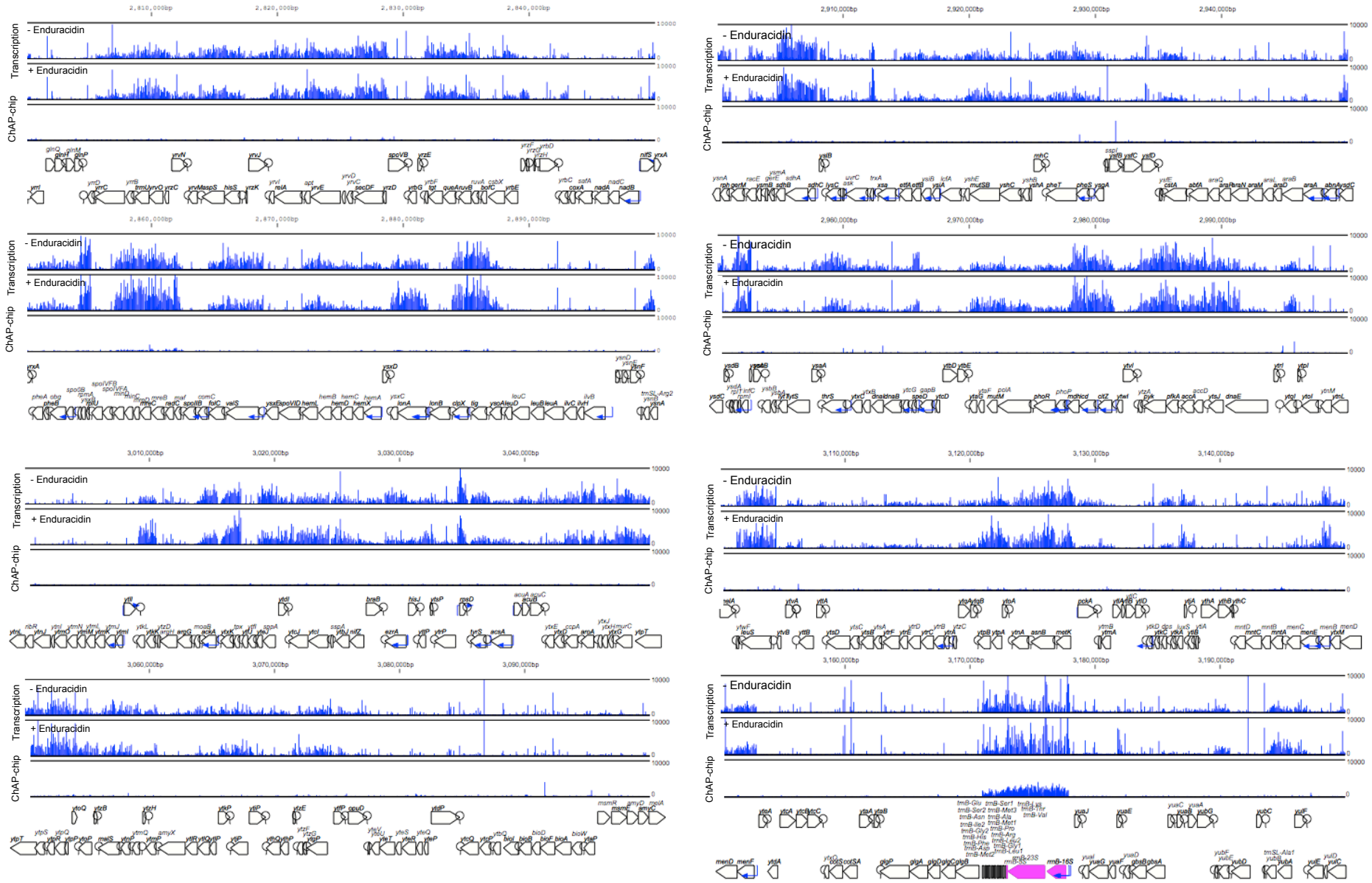

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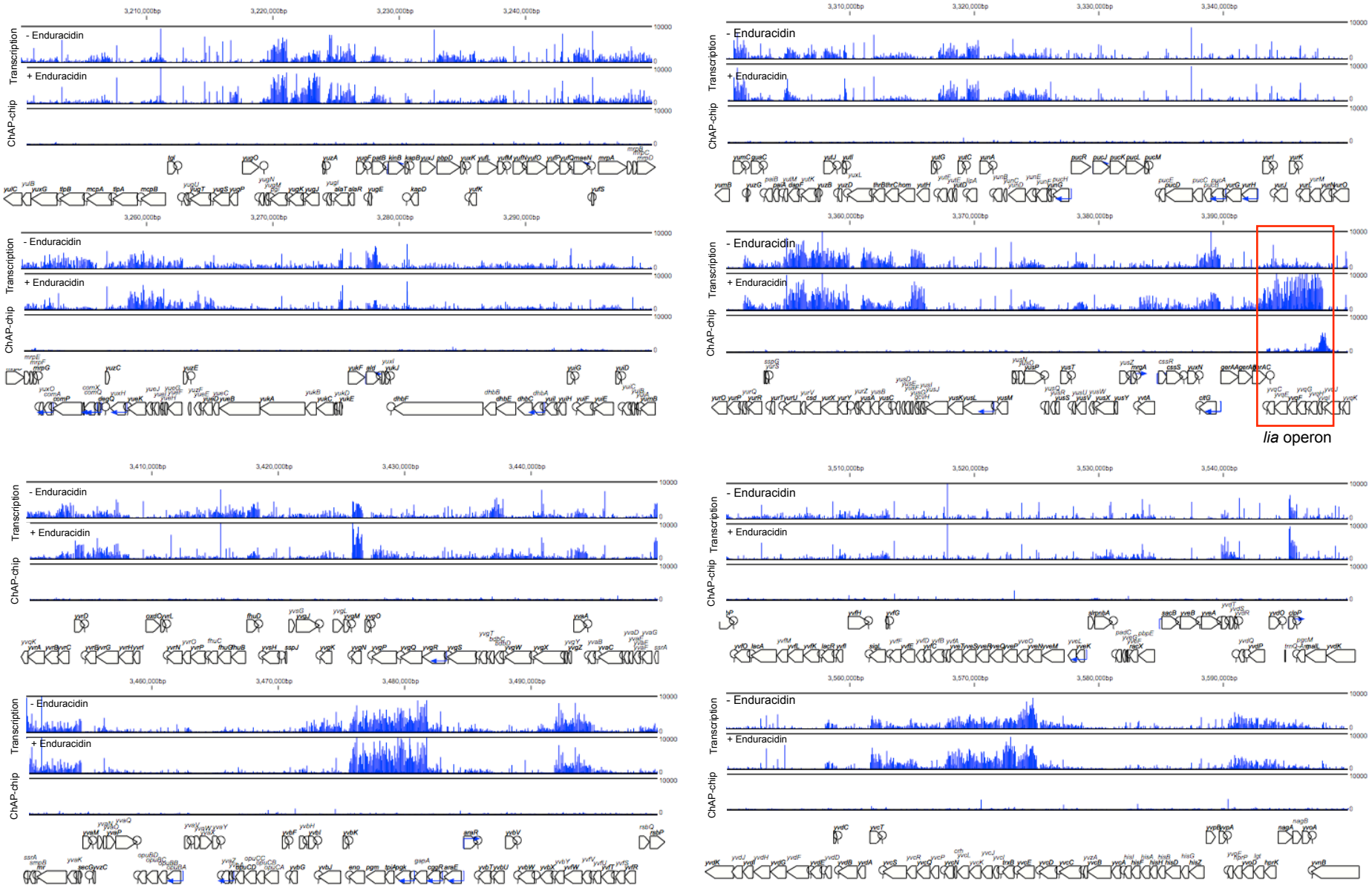

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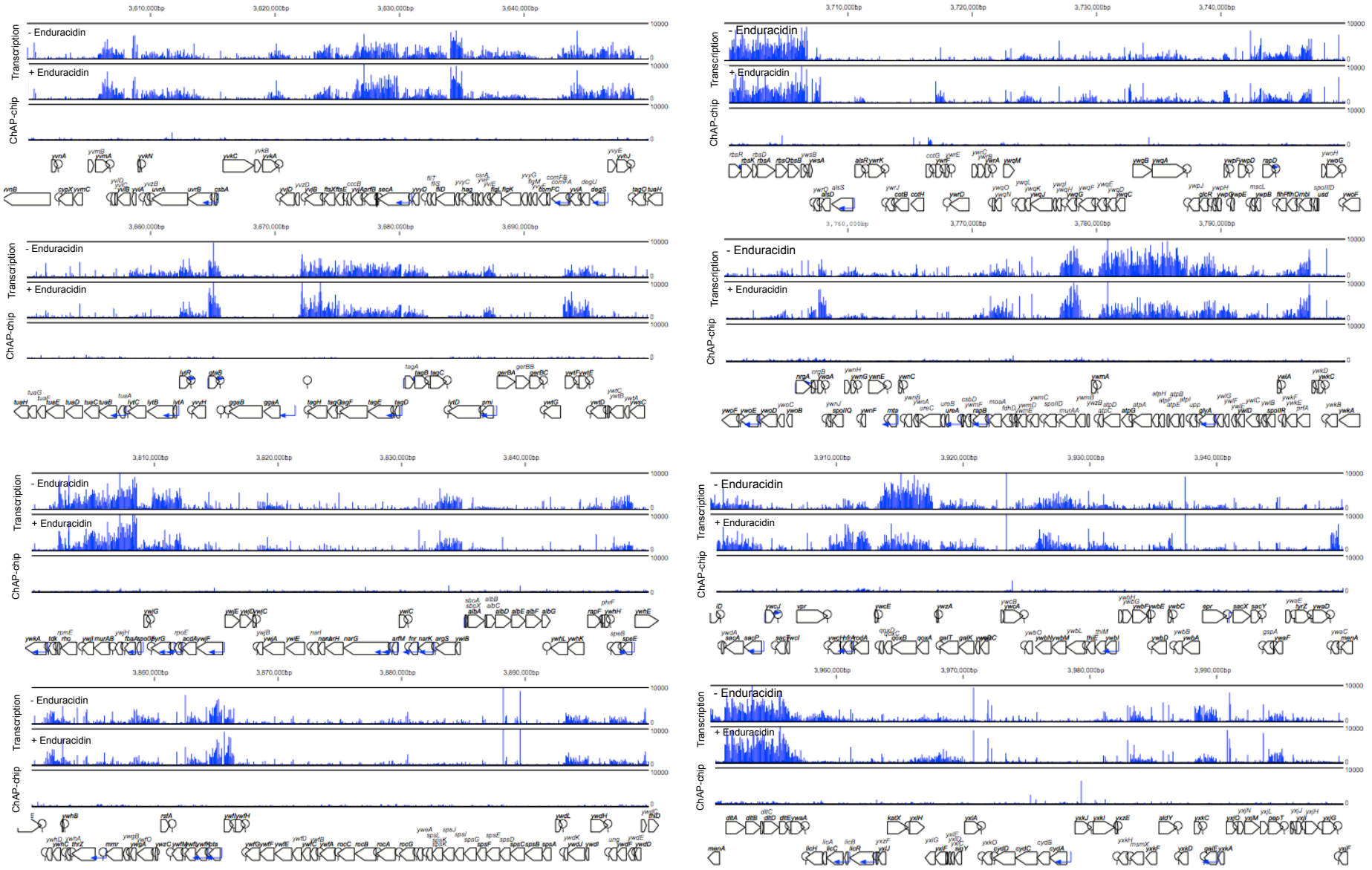

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