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The *tcrB* gene is part of the *tcrYAZB* operon conferring copper resistance in *Enterococcus faecium* and *Enterococcus faecalis*

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The plasmid-localized *tcrB* (transferable copper-resistance gene B) gene from *Enterococcus faecium* was identified to be part of an operon called the *tcrYAZB* operon, which has a genetic organization similar to the *copYZAB* copper-homeostasis gene cluster from *Enterococcus hirae*. Putative promoter (P_{tcr})- and repressor-binding sites highly similar to the *E. hirae* *cop*-promoter region were identified upstream of the *tcrYAZB* genes. The P_{tcr} promoter was cloned in both the absence and the presence of the proximal repressor-encoding *tcrY* gene into a promoter-probe vector. Induction of the promoter was shown in liquid growth medium containing increasing concentrations of copper sulphate. To determine the growth advantage conferred by the *tcrYAZB* genes in a copper environment, a *tcr*-deletion mutant was isolated, and its growth was compared with that of its copper-resistant ancestor (strain A17sv1) in sublethal concentrations of copper sulphate. A competition assay using these two isogenic strains showed that copper sulphate concentrations of 3 mmol l^{-1} and above are sufficient to select for copper resistance.

INTRODUCTION

Copper is an essential trace metal to all living organisms, where it serves as a cofactor for a large number of enzymes. Therefore, all living cells have developed homeostatic mechanisms to ensure adequate levels of copper within the cell. One of the most studied bacterial copper homeostasis mechanisms is the *copYZAB* operon of the Gram-positive bacterium *Enterococcus hirae*, recently reviewed by Solioz & Stoyanov (2003). This operon encodes four proteins (CopY, CopZ, CopA and CopB) working in concert to maintain tolerable levels of copper inside the cell (Lu & Solioz, 2002). CopA and CopB are two membrane-localized CPx-type ATPases involved in Cu^{2+} trafficking across the membrane. CPx-type ATPases (also called P-type ATPases) are soft-metal transporters, which all contain a CPC or CPH motif in their active site (Solioz & Vulpe, 1996). The CopA protein is probably an influx pump (Odermatt & Solioz, 1995; Wunderli-Ye & Solioz, 2001), while CopB is responsible for copper efflux (Solioz & Odermatt, 1995). CopY is a transcriptional repressor of the copper-responsive promoter located upstream of the four genes (Strausak & Solioz, 1997). It affects the expression of the downstream genes through zinc-dependent binding to two regulatory operator sites overlapping the promoter (Strausak & Solioz, 1997). The central recognition site of the operator has been suggested to be the so-called *cop* box, which has the consensus sequence TACANNTGTA. This sequence is found in *cop* promoters from several different Gram-positive organisms, including *Lactococcus lactis* and *Streptococcus mutans* (Portmann *et al.*, 2004), while a slightly modified sequence is present in *Bacillus subtilis* (TACGNNGGTA). When copper is in excess, copper ions replace the zinc atom embedded inside CopY, and DNA binding is abolished (Cobine *et al.*, 1999). The fourth protein encoded by the *copYZAB* operon is CopZ, a copper chaperone responsible for Cu^{2+} trafficking in the periplasm. Here, it transfers Cu^{2+} to CopY (Cobine *et al.*, 1999, 2002), and possibly also to CopB for transport to the exterior (Elam *et al.*, 2002).

Homeostatic mechanisms like the *copYZAB* system are rarely able to handle artificially elevated concentrations of copper. In response to toxic levels of copper, plasmid-borne copper resistance mechanisms are often employed. The *pco* system from *Escherichia coli*, and the *cop* system from *Pseudomonas syringae* pv. *tomato* (Brown *et al.*, 1995; Lee *et al.*, 2002), are well-known model systems of transferable copper resistance in Gram-negative bacteria (Bender & Cooksey, 1986; Mellano & Cooksey, 1988). However, these systems do not involve CPx-type ATPases. The *tcrB* (transferable copper-resistance gene B) gene is, to date, the only plasmid-encoded and transferable CPx-type copper ATPase gene described. The *tcrB* gene has been described in *Enterococcus faecium* (Hasman & Aarestrup, 2002) and *Enterococcus faecalis* (Aarestrup *et al.*, 2002), where it confers copper resistance. Strains harbouring the *tcrB* gene have an MIC of 24 mmol l^{-1} for $CuSO_4$, whereas strains lacking the *tcrB* gene have a MIC of 2–8 mmol l^{-1} (Hasman & Aarestrup,

2002). The prevalence of *tcrB* in Denmark among *E. faecium* is especially high among isolates from pigs compared with other reservoirs: 46–79 % of pig isolates examined between 1997 and 2003 were copper resistant (Hasman & Aarestrup, 2005). The most likely explanation for this high prevalence is the use of $CuSO_4$ as a growth-promoting agent for pigs: piglets in Denmark and most of the European Union receive 175 p.p.m. $CuSO_4$, and slaughter animals receive 35 p.p.m. $CuSO_4$ in their feedstuff. A relationship between copper resistance, and glycopeptide and macrolide resistance in *E. faecium* has been established previously (Hasman & Aarestrup, 2002, 2005). Therefore, a closer examination of the mechanisms and selective concentrations responsible for development of copper resistance in *E. faecium* is needed.

This paper identifies the *tcrB* gene to be part of a previously uncharacterized operon called the *tcrYAZB* operon, with a genetic organization highly similar to the *copYZAB* operon from *E. hirae*. This operon has been further characterized, and its ability to select for copper resistance has been examined.

METHODS

Strains. The copper-resistant *E. faecium* strain A17sv1, containing the *tcrYAZB* gene cluster on a wild-type plasmid, and isolated from a healthy pig in 1995, was used throughout this study (Hasman & Aarestrup, 2002). A17sv1 is also resistant to vancomycin, mediated by the *vanA* gene cluster, and erythromycin, mediated by the *erm*(B) gene. As recipients for promoter fusion constructs, the following strains were used: the copper-sensitive *E. faecalis* strain JH2-2 RF (Dunny & Clewell, 1975), and the copper-resistant *E. faecalis* strain 9831021-2, carrying the *tcrYAZB* gene cluster, and isolated from a healthy pig in 1998 as part of the DANMAP surveillance programme (Danish Integrated Antimicrobial Resistance Monitoring and Research Programme, 2000).

PCR and sequencing. Sequencing of the *tcr* operon was completed by inverse PCR, as described by Hui *et al.* (1998). In short, two primers (P511, 5′-GGA AAG GCA ACT GAA TAT CC-3′, and P560, 5′-GCC GTC TTG ATG TCA CTT TC-3′) were designed to read downstream and upstream, respectively, of the previously sequenced *tcrB* gene (Hasman & Aarestrup, 2002). Plasmid DNA from A17sv1 was purified, and 20 µl (50 ng μl^{-1}) of this was digested separately with a series of different restriction enzymes that are known not to cut inside the *tcrB* gene. The digested DNA was purified using a GFX PCR DNA and gel band purification kit (Amersham Biosciences), and eluted in 50 µl double-distilled water. T4 DNA ligase (Invitrogen) was added to this 50 µl eluate, and the DNA was religated to generate circular DNA molecules. Then, 2 µl of the ligation mix was used for PCR with the two primers. In cases where a single PCR product was generated, this was sequenced, and two new primers were designed based on this new sequence, until the complete sequence of the *tcr* gene operon was obtained. The complete sequence of *tcrYAZB* has been submitted to GenBank (accession no. AY048044).

Cycle sequencing of the PCR products was carried out according to the manufacturer's instructions, using an AmpliTaq dye terminator kit and a 373A automatic sequencer (Applied Biosystems/Perkin Elmer). The Vector NTI suite v8.0 (Invitrogen) was used to assemble sequencing fragments.

Construction of promoter fusions to pAK80. The computer program Winseq32 (a kind gift from Flemming G. Hansen, Biocentrum-DTU, Denmark) was used to identify a potential promoter upstream of *tcrY*. Based on the sequencing result of the complete *tcr* operon described above, two sets of primers were designed using the computer program Vector NTI suite v8.0. The first set of primers was designed to amplify a 220 bp DNA fragment predicted to carry the copper-responsive promoter P_{tcr}, including putative regulatory binding sites. The forward primer (P1: 5′-CCC AAG CTT ACA GAG AAG TGT CCG ACG AAC C-3′) was designed to carry a *Hin*dIII site, and the reverse primer (P2: 5′-CCC GGA TCC TCA TAT TCT CTC CCC CTT TCG TT-3′) was designed to contain a *Bam*HI site, for cloning into the erythromycin-resistant promoter-probe vector pAK80 containing the promoterless β-galactosidase genes *lacL* and *lacM* behind a multicloning site (Israelsen *et al.*, 1995), thus generating the plasmid pHHA213. The second set of primers was designed to amplify a 679 bp DNA fragment carrying precisely the same region as above, as well as the downstream putative regulatory gene *tcrY*. Here, the forward primer (P3: 5′-CGC CTC GAG ACA GAG AAG TGT CCG ACG AAC CA-3′) was designed to contain an *Xho*I site, and the downstream primer (P4: 5′-CTC GGA TCC TCG CTC CTT ATT CTC CAT GAT GAT G-3′) to contain a *Bam*HI site for insertion into pAK80, which generated the plasmid pHHA218. Amplification of the DNA fragments was done using the EXPAND High Fidelity^{PLUS} PCR system (Roche) under standard PCR conditions: 94 °C for 3 min, then 25 cycles (94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min), and finally 72 °C for 10 min. The inserts of both plasmids were sequenced to exclude PCR-generated mutations.

β-Galactosidase assay. pAK80, pHHA213 and pHHA218 were electroporated into the copper- and erythromycin-sensitive *E. faecalis* strain JH2-2 RF as well as the copper-resistant and erythromycin-sensitive *E. faecalis* strain 9831021-2, as described by Dunny *et al.* (1991), and selected on 16 µg erythromycin ml^{-1}. A single colony from each electroporated strain was inoculated into BHI broth (Oxoid) containing 16 µg erythromycin ml^{-1}, and grown overnight at 30 °C, with gentle shaking (125 r.p.m.). A 100 µl volume of each culture was reinoculated into fresh preheated BHI broth containing 0, 4, 8, 12 and 16 mmol $CuSO_4$ l^{-1} (pH 7), and grown at 30 °C, with gentle shaking until an OD_{600} of 0·3–0·4 was reached for each culture. Then, three sets of 2 ml culture samples were collected from each concentration, and β-galactosidase activity was measured for each set, as described by Miller (1992).

Creation of the *vanA–tcr* deletion mutant. The A17sv1 strain was grown overnight at 37 °C in BHI broth containing 5 µg novobiocin ml^{-1} (Sigma-Aldrich), in an attempt to cure the plasmid carrying the *tcr* genes. Screening of a large number of colonies led to an isolate (A17sv1-34) that had lost the copper-resistance and vancomycin-resistance phenotypes. The loss of the *tcr* and *van* genes was confirmed by Southern blot analysis using specific probes directed towards *tcrB* and *vanA*, respectively. The clonal relationship to A17sv1 was confirmed by PFGE using *Sma*I digestion of chromosomal DNA, as described by Aarestrup (2000).

Conjugation. In order to ensure plasmid location of the resistance genes, plasmids from A17sv1 and A17sv1-34 were transferred by filter-mating to the plasmid-free *E. faecium* recipient BM4105RF (rifampicin- and fusidic-acid-resistant), as described by Clewell *et al.* (1985). Transconjugants were selected on BHI agar containing 16 µg erythromycin ml^{-1}, 25 µg rifampicin ml^{-1} and 25 µg fusidic acid ml^{-1}.

Plasmid purification. Plasmids were isolated using Qiagen Plasmid Midi kit (Qiagen), as described previously (Hasman & Aarestrup, 2002).

Growth curves. The A17sv1 and the A17sv1-34 (*tcr*) strains were grown on BHI agar (Oxoid) containing 16 µg erythromycin ml^{-1}, at 37 °C overnight. The next day, the two strains were inoculated into

10 ml BHI, and grown overnight at 37 °C, with gentle shaking. The OD_{600} was measured, and the cultures were diluted approximately 3000-fold to an OD_{600} of 0·001 in preheated BHI broth containing 0, 1, 2, 3 and 4 mM $CuSO_4$ (pH 7·0). Then, 300 µl of each culture was dispensed into separate wells of a 100-well Bioscreen microwell plate. The Bioscreen microwell plate was inserted into a Bioscreen C apparatus (Growth Curves AB), and analysed using the software Research Express (Transgalactic). Hardware settings were as follows: temperature, 37 °C; continuous shaking (medium, 80 steps); measurement of OD_{492} every 12 min for 18 h. Each strain was tested in the same media at least four times, and the mean generation time (t_{gen}) was calculated.

Competition assay. A growth competition assay was done between A17sv1 and A17sv1-34 in different concentrations of $CuSO_4$. Overnight cultures of the two strains were mixed with a surplus of the copper-sensitive strain A17sv1-34 (in a 100 : 1 ratio), and 100 µl of this mixed culture was then transferred to eight different flasks. These flasks contained 25 ml preheated BHI broth with 0, 1, 2, 3, 4, 8, 12 or 16 mmol $CuSO_4$ l^{-1}(pH 7), and they were incubated at 37 °C, with gentle shaking. After 8 h, 100 µl of each culture was transferred to a fresh flask containing 25 ml preheated medium supplemented with the same copper concentration as the previous flask. This was repeated three times, leading to a total of 32 generations of growth. The cell suspension from the last flask was diluted by an appropriate factor of between 10^5- and 10^7-fold, and then plated onto BHI agar containing 16 µg erythromycin ml^{-1}, for incubation overnight at 37 °C. From each concentration, 100 colonies were picked randomly, and streaked onto two sets of BHI agar containing either 16 mmol $CuSO_4$ l^{-1}or 16 µg erythromycin ml^{-1}, and incubated overnight at 37 °C. From these cultures, the ratios of copper-resistant bacteria were calculated. Furthermore, the presence of the *tcrYAZB* genes in the copper-resistant population was tested by PCR, as previously described (Hasman & Aarestrup, 2002).

RESULTS AND DISCUSSION

Organization of the *tcr* gene operon

Sequencing the flanking regions of the previously sequenced *tcrB* gene identified on a naturally occurring plasmid from the copper-resistant *E. faecium* isolate A17sv1 in search of its corresponding promoter revealed the *tcrB* gene to be part of an operon consisting of four ORFs. Computer analysis suggested a putative promoter (P_{tcr}) to be located upstream of the first ORF of this operon (*tcrY* in Fig. 1). The individual ORFs and the promoter showed strong homology to the well-characterized copper homeostasis *copYZAB* operon from *E. hirae* (Table 1). By analogy to the *E. hirae* counterparts, the genes of the *tcr* operon were thus named *tcrY*, *tcrA*, *tcrZ* and *tcrB*, respectively (Fig. 1). The first gene of the operon, *tcrY*, was a 453 bp gene encoding a 151 aa putative protein called TcrY. TcrY was homologous to the CopY repressor from *E. hirae*, and contained a $CXCX_4CXC$ in the C-terminal part of the protein. This domain has been suggested to be the zinc- and copper-binding domain common to all CopY-like repressors (Lu & Solioz, 2002), and thus indicates that TcrY is involved in expression control of the operon.

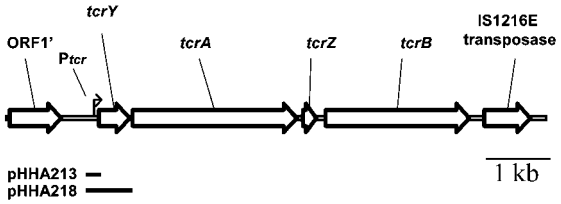

Fig. 1. Organization of the *tcrYAZB* operon including the flanking regions. The different DNA elements are described in the text. The location of the *tcr* promoter (P_{tcr}) is indicated by an arrow. The parts of the sequence that were cloned into pAK80 to generate the two plasmids pHHA213 and pHHA218 are indicated to the left with horizontal lines.

Table 1. Nucleotide and protein identities between the genetic elements of the *tcrYAZB* operon and the same elements from the *copYZAB* operon of *E. hirae*

Values are percentages. P_{cop} and P_{tcr} are the *cop* and *tcr* promoters, respectively.

Genetic structure	DNA	Protein
P_{cop} vs P_{tcr}	70·0	NA
copY vs *tcrY*	56·2	44·4
copA vs *tcrA*	51·9	49·3
copZ vs *tcrZ*	42·7	27·5
copB vs *tcrB*	56·9	46·3

NA, Not applicable.

The *tcrA* gene (2433 bp) encoded a putative copper-influx CPx-type ATPase called TcrA (811 aa). TcrA showed strong homology to CopA from *E. hirae* (Table 1), and contained all features believed to identify a CPx-type copper transporter (Solioz & Stoyanov, 2003). These features include: (1) two CXXC motifs in the N-terminal part of the protein believed to be involved in the initial contact with CopZ; (2) a TGES phosphatase domain; (3) an intramembranous CPC trafficking motif; (4) a DKTGT aspartyl kinase domain; (5) a conserved HP motif, 40 aa downstream of the aspartic acid of the aspartyl kinase domain; and (6) the ATP-binding consensus domain (GDGINDAP).

tcrZ was a 204 bp gene encoding a putative chaperone protein called TcrZ (68 aa), with homology to other copper chaperones, including CopZ from *E. hirae*. It also contained the CXXC motif in the N-terminus, which is normally found in CopZ-like chaperones. The last ORF in the operon, *tcrB*, has been described previously as encoding a copper efflux pump (TcrB), homologous to CopB from *E. hirae* (Hasman & Aarestrup, 2002).

The close homology to the *copYZAB* operon of *E. hirae* makes the structural relationship evident, and gives an indication of the function of the plasmid-located *tcrYAZB* genes within the cell, but the origin of the operon remains elusive, as the order of the individual genes is not the same as the *cop* genes in *E. hirae*. The chaperone *tcrZ* is located between the *tcrA* and *tcrB* genes in the *tcr* operon, whereas

the chaperone *copZ* is located between the repressor *copY* and *copA* in *E. hirae*. Similar location of the *copZ* gene after the *copA* gene is seen among copper-homeostasis genes from *S. mutans* and *Streptococcus gordonii* (Vats & Lee, 2001; Mitrakul *et al.*, 2004), which could give a hint to the possible origin of the plasmid-located *tcr* operon, especially as the DNA and protein homology to the streptococcal *cop* gene clusters from *S. mutans* and *S. gordonii* are only slightly less than the homology to *E. hirae* (data not shown).

A truncated ISS1-type transposase (ORF1′ in Fig. 1) was located upstream of the *tcr* promoter, and an IS*1216E* element was located downstream of the four *tcr* genes, indicating the termination of the operon (Fig. 1). This was further supported by the fact that the intergenic region between *tcrB* and the IS*1216E* element contained a strong dyad symmetry region (ΔG −21·4 kcal mol^{-1}; 89·5 kJ mol^{-1}) able to form a loop structure, which could function as a factor-independent transcriptional terminator.

The *tcrYAZB* genes are transcribed from a promoter regulated by $CuSO_4$

A putative promoter structure (P_{tcr}) was located immediately upstream of the *tcrY* gene (Fig. 1), with the −35 and −10 boxes (underlined in Fig. 2) separated by a 16 bp spacer region. Again, this region showed strong homology to the promoter region of the *cop* promoter from *E. hirae* (Table 1), including the −35 box, the −10 box and the two repressor operator half-sites (indicated with arrows in Fig. 2). Two imperfect *cop* boxes were located within these repressor operator sites (indicated with grey in Fig. 2). As the *cop* box has been defined using CopY from *E. hirae*, this could indicate that TcrY has a slightly altered recognition site compared with CopY. This makes biological sense, as TcrY could otherwise interfere with the normal regulation of copper homeostasis.

Taken together, the data presented above suggest that the *tcr* operon is regulated in a similar way to the *cop* operon from *E. hirae*. Therefore, a 220 bp DNA fragment (indicated in Fig. 1), expected to carry only the copper-responsive promoter, including putative regulatory half-sites, was cloned into the promoter-probe vector pAK80, containing a promoterless β-galactosidase reporter cassette, to generate the plasmid pHHA213. A second plasmid called pHHA218 was created to contain precisely the same DNA region, as well as the *tcrY* gene downstream of the promoter (Fig. 1). These two plasmids, as well as the vector control plasmid pAK80, were inserted into the copper-sensitive (MIC 6 mmol $CuSO_4$ l^{-1}) plasmid-free *E. faecalis* strain JH2-2 RF. The β-galactosidase activity from the putative promoter (pHHA213), and the effect of the presence of the putative repressor (pHHA218), were examined in liquid growth medium without (0 mmol l^{-1}) and with (4 mmol l^{-1}) supplementation of $CuSO_4$ (Table 2). As can be seen, expression from the promoter in the absence of the repressor protein TcrY (pHHA213) was strong both in the absence and presence of $CuSO_4$. In contrast to this, expression was almost completely repressed by the presence of the TcrY protein (pHHA218) in BHI broth without supplementation of $CuSO_4$. Upon induction with 4 mmol $CuSO_4$ l^{-1}, the cells carrying pHHA218 showed an increase in β-galactosidase activity of 536 %. This indicates that TcrY is a specific repressor of the P_{tcr} promoter, and that expression can be de-repressed by addition of copper to the medium. However, the expression level at 4 mmol $CuSO_4$ l^{-1} was far from the full expression seen for the cells harbouring pHHA213. This is not surprising, as the *tcr* operon is capable of providing copper resistance up to 24 mM $CuSO_4$ in *E. faecalis* (Aarestrup & Hasman, 2004), and a graduated response to copper would be expected.

Table 2. Specific activity (Miller units) of promoter fusions to the *lacLM* genes of the promoter probe vector pAK80 (vector control) in the copper-sensitive *E. faecalis* strain JH2-2RF

Plasmid	$CuSO_4$ concn		Change in activity*
	0 mmol l^{-1}	4 mmol l^{-1}	
pAK80	0·22±0·01	0·046±0·008	−80 %
pHHA213	120±16	64±7·5	−47 %
pHHA218	0·280±0·03	1·5±0·3	+536 %

*Relative change in activity in 4 mM $CuSO_4$ compared with 0 mM $CuSO_4$. −, decrease in activity; +, increase in activity.

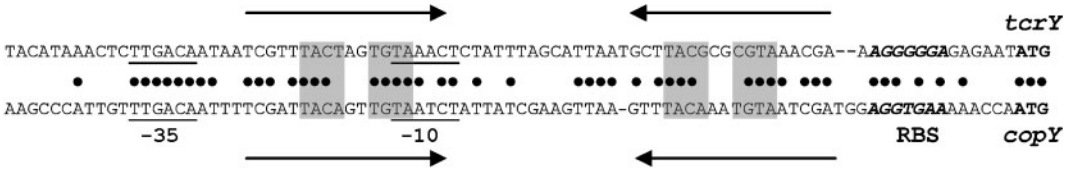

Fig. 2. Nucleotide sequence alignment of the *tcr* promoter (top string) and the *cop* promoter of *E. hirae* (bottom string). ●, Identical nucleotides. The −35 and −10 boxes of the *cop* promoter are underlined, and the most likely candidates of the *tcr* promoter are indicated in a similar way. At the far right, the initiation codons of the *tcrY* and *copY* genes are in bold, and optimal ribosome-binding sites (RBS) (in bold and italic) are upstream of the initiation codon. Arrows mark the inverted repeats of the repressor-binding sites, and grey shading indicates suggested *cop* boxes.

Since experiments with JH2-2 RF in concentrations of $CuSO_4$ higher than 4 mmol l^- were unsuccessful due to the toxic effect of the copper, a *tcr*-positive copper-resistant (MIC $\geqslant$24 mmol $CuSO_4$ l^{-1}) and erythromycin-sensitive (MIC 1 μg ml^{-1}) *E. faecalis* wild-type isolate, 9831021-2, was selected for electroporation of the same three plasmids as described above. As a side effect of the copper-resistance phenotype needed for the induction at higher concentrations of Cu^{2+}, this configuration introduced the *tcrYAZB* genes to the cells *in trans*, thus delivering the TcrY repressor to both pHHA213 and pHHA218. The β-galactosidase assay was repeated with this new bacterial host in 0, 4, 8, 12 and 16 m mmol $CuSO_4$ l^{-1}. As can be seen in Fig. 3, virtually no expression occurred in the absence of $CuSO_4$ (the specific β-galactosidase activity was below 1 Miller unit for the three plasmid constructs). At 4 mmol $CuSO_4$ l^{-1} weak expression from pHHA213, but not pHHA218, was seen. This difference was probably caused by the higher gene dose of TcrY in the latter cells, which caused a tighter repression of the promoter. As the copper concentration increased to 8 and 12 mmol l^{-1}, expression from both pHHA213 and pHHA218 increased further, and, at 16 mmol l^{-1}, expression from both constructs increased significantly (614 % and 1465 % when the copper concentration was increased from 12 to 16 mmol l^{-1} for pHHA213 and pHHS218, respectively). This confirmed that TcrY was fully able to repress the P_{tcr} promoter, and showed that it could be de-repressed by higher concentrations of Cu^{2+}.

The *tcr* promoter contained two inverted repeats at exactly the same position relative to the −35 and −10 boxes as the inverted repeats that the CopY repressor has been shown to bind to in the *cop* promoter in *E. hirae* (Fig. 2). It is therefore likely that these inverted repeats serve as repressor binding sites for TcrY, but this still remains to be tested.

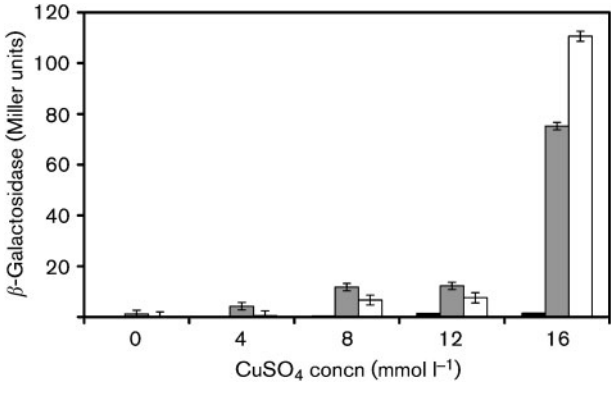

Fig. 3. Expression levels (in Miller units) of the *tcr* promoter fusions to *lacLM* from pAK80 in different concentrations of $CuSO_4$ when the *tcrYAZB* gene cluster is presented *in trans*. Black bars, pAK80 in *E. faecalis* 9831021-2; grey bars, pHHA213 in *E. faecalis* 9831021-2; white bars, pHHA218 in *E. faecalis* 9831021-2. The 95 % confidence intervals based on three independent measurements are indicated on each bar.

Furthermore, the experiment above shows that the P_{tcr} promoter suggested to exist within the cloned fragment of pHHA213 is able to promote Cu^{2+}-induced expression of the *tcr* genes at Cu^{2+} concentrations that are toxic to cells lacking the *tcr* genes.

Construction of a *tcr*-deletion mutant

A *tcr*-deletion mutant (A17sv1-34; MIC 6 mmol $CuSO_4$ l^{-1}) of the copper-resistant *E. faecium* isolate A17sv1 was isolated. The A17sv1-34 strain had retained its erythromycin-resistance phenotype (MIC $\geqslant$32 μg ml^{-1}), indicative of a deletion in, rather than curing of, the plasmid. Conjugational transfer to the plasmid-free *E. faecium* recipient BM4105RF using erythromycin as selective marker confirmed this. Plasmid purifications of the A17sv1 and A17sv1-34 strains were compared by RFLP analysis using *Eco*RI and *Pvu*II, and then subjected to Southern blotting to ensure complete deletion of the *tcr* and *vanA* genes. Based on the RFLP analysis, the size of the deletion could be estimated to approximately 75 kb, leading to a total plasmid size of approximately 100 kb (data not shown).

The *tcr* genes confer a growth advantage in sublethal concentrations of $CuSO_4$

The generation times (t_{gen}) of the A17sv1-34 mutant in different concentrations of $CuSO_4$ were compared with the those of the wild-type (Fig. 4). At $CuSO_4$ concentrations between 0 and 2 mmol l^{-1}, the doubling times of the two strains were indistinguishable, at around 30 min, but at $CuSO_4$ concentrations equal to or above 3 mmol l^{-1}, the t_{gen} of both cultures was influenced by the metal ions. However, the growth of the *tcr* mutant was significantly more affected than the wild-type strain, with an approximately 25 % reduction in the growth rate at 4 mmol $CuSO_4$ l^{-1} for the *tcr* mutant compared with the A17sv1 wild-type strain. At $CuSO_4$ concentrations of 6 mmol l^{-1} and above, only the A17sv1 strain was able to grow. So, even at low

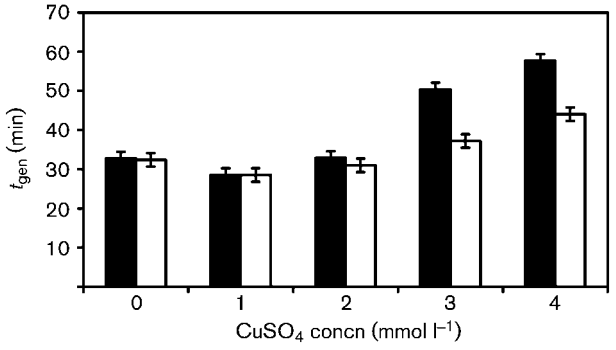

Fig. 4. Generation times (t_{gen}) of the copper-sensitive *E. faecium* isolate A17sv1-34 (black bars) and its isogenic copper-resistant ancestor A17sv1 (white bars) in different concentrations of $CuSO_4$. 95 % confidence intervals based on four independent measurements are indicated on each bar.

levels of $CuSO_4$, where both strains were able to grow, and where the *tcr* promoter was only induced at a low level in the promoter fusion experiment described above, copper did impose a significant effect on the growth of the copper-sensitive strain relative to the copper-resistant strain.

Low levels of $CuSO_4$ select for copper-resistant bacteria

In order to test whether this difference in growth inhibition did have a selective effect on the copper-resistant strain compared with the copper-sensitive strain, the two bacteria were mixed in a 1 : 100 ratio favouring the copper-sensitive A17sv1-34 strain, and added to liquid medium supplemented with different amounts of $CuSO_4$ between 0 and 16 mmol l^{-1}. The mixed culture was then grown in fresh medium repeatedly for three consecutive days, corresponding to approximately 32 generations. A17sv1 did not have a selective growth advantage in 0, 1 and 2 mmol $CuSO_4$ l^{-1}, as the percentage of copper-resistant bacteria did not change from the initial 1 %. This is in good agreement with the growth experiments of the individual strains described above, where the doubling times did not differ significantly below 3 mmol $CuSO_4$ l^{-1}. At 3 mmol $CuSO_4$ l^{-1}, the fraction of copper-resistant isolates in the mixed culture rose to 24 %. PCR confirmed the presence of the *tcrYAZB* genes in these copper-resistant isolates. This was exactly the copper concentration at which the two strains differed significantly in their growth rates. Therefore, there was good agreement between the two experiments. Based on the differences in generation times, it is likely that the copper-resistant population would have dominated completely if the competition assay had been continued. At copper concentrations above 3 mmol l^{-1}, this selection of copper-resistant bacteria was even more pronounced, as 81 % were resistant at 4 mmol $CuSO_4$ l^{-1}, and there was eventually complete domination (100 %) of the A17sv1 strain when the mixed cultures were grown in the presence of copper concentrations (8 or 16 mmol l^{-1}) that were above the MIC of the *tcr* mutant.

Based on the observations described above, the question is then, can the use of $CuSO_4$ in concentrations of up to 175 p.p.m. in production animals select for Cu^{2+} resistance among *E. faecium*? Interestingly, 175 p.p.m. $CuSO_4$ is equal to 2·8 mmol l^{-1} Cu per kg feedstuff. This concentration is comparable to the selective concentration of 3 mM found in this study, and could therefore lead to selection of copper-resistant *E. faecium* in the gut of piglets fed 175 p.p.m. $CuSO_4$. However, factors in the gut, such as the pH, copper speciation, adsorption and complex formation to organic material, have an influence on the actual copper concentration. Furthermore, it is unlikely that the concentration in the intestine remains the same as it is in the feed initially, because feed components are removed from the feed for growth of the animal, and also because an unknown volume of water is added during the digestion process. The selective copper concentration found in this study can therefore only serve as an indication for the effect of adding high doses of copper to the feed used in pig production.

Based on the results presented above, the five times lower concentration given to slaughter animals (35 p.p.m.) is more likely to be below the selective concentration, and therefore less likely to select for copper resistance. This could explain why we find a high level of copper-resistant bacteria, but not full resistance, among *E. faecium* isolated from slaughter pigs as part of the DANMAP programme (Hasman & Aarestrup, 2005).

Since copper resistance is closely linked to resistance to erythromycin (a macrolide) and vancomycin (a glycopeptide) in *E. faecium* from pigs in Denmark, the results presented here cannot exclude the possibility that addition of $CuSO_4$ to the feed can co-select for these antibiotic resistances. Data regarding co-selection of macrolide and glycopeptide resistance, as well as the level of copper-resistant *E. faecium* in piglets fed 175 p.p.m. $CuSO_4$ to evaluate such a hypothesis, do not exist, but this is currently under examination in animal feeding studies.

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