Supplemental Methods

Bacterial strains. A total of 408 *E. faecalis* strains isolated over 30 years from diverse locations (Argentina, Belgium, Canada, Chile, China, France, Lebanon, Spain, Thailand, UK, and USA) were included. These isolates include ones from endocarditis (82), urine (92), other clinical specimens (121), stool isolates from hospitalized patients (58), community-derived human fecal isolates (33), and animal isolates (22). The sources of other clinically-derived strains include blood, bile, bone, catheters, cervix, cerebrospinal fluid, placenta, peritoneal fluid, sputum, and several types of wounds, among others. All the *E. faecalis* isolates used in this study were initially identified to species level by biochemical tests and were confirmed by colony hybridization (1) using an intragenic *ace* probe (2, 3). To study the presence of *ebpA*, *ebpB*, and *ebpC*, respective intragenic fragments were amplified (using primer pairs Ef1091F and Ef1091R, Ef1092F and Ef1092R, and, Ef1093F and Ef1093R, respectively (Supplemental Table 2)) by PCR, labeled with ^{32}P and used as probes for colony hybridization.

Construction of srtC deletion mutant of E. faecalis OG1RF. To delete the 855-bp *srtC* gene of OG1RF, a crossover PCR deletion product containing the *srtC* upstream and downstream regions was made in two steps as described earlier (4). The crossover PCR product was purified, digested with PstI and XbaI, and ligated with similarly digested pTEX4577. A correct construct, designated as pTEX5468, was then introduced into electrocompetent cells of OG1RF and the cells were then plated on kanamycin to select for single crossover integration. Primer sets Ef1094delUpF and T7 as well as Ef1094delDnR and T3 (Supplemental Table 2) were used for verifying integration into the left or right regions, respectively. One of the recombinants was picked and was grown for six daily serial passages at 37°C. The culture from the sixth passage was serially diluted and plated at 37°C on non-selective media. To detect plasmid excision by

double crossover recombination, these master plates were then replica plated to BHI plates and BHI plus kanamycin plates to identify colonies that had lost the kanamycin resistance gene of the vector. The deletion mutant was differentiated from a wild-type revertant by PCR with primers Ef1094delUpF and Ef1094delDnR and the strain identity was confirmed by PFGE. One of these colonies with the correct deletion was designated as TX5470 (OG1RFΔ*srtC*). Southern hybridizations using the deleted fragment of *srtC* as a probe and sequencing of the Ef1094delUpF and Ef1094delDnR PCR product with primer Ef1093DownFa (Supplemental Table 2) confirmed the correct deletion.

Construction of ebpA allelic replacement mutation. Construction of TX5475 involved deletion of *ebpA* with substitution of a kanamycin resistance gene cassette in its place. The crossover PCR deletion products that were used to delete the *ebpA* gene from OG1RF contained the *ebpA* upstream and downstream regions joined by a kanamycin resistance cassette between them. The crossover PCR product was cloned into pTEX4594 (Supplemental Table 1). A correct construct, designated as pTEX5473, was then introduced into electrocompetent cells of OG1RF and the cells were then plated on kanamycin and erythromycin to select for single crossover integration. Appropriate primer sets (Supplemental Table 1) were used for verifying integration into the left or right regions, respectively. One of the integrants grown for several serial passages was serially diluted and plated at 37°C on non-selective media. To detect plasmid excision following a second crossover recombination, replica plating was used. The deletion mutant TX5475 (OG1RFΔ*ebpA*) was confirmed by PFGE, Southern hybridization, and sequencing, as above.

Supplemental Table 1 Bacterial strains and plasmids used in this study

Strains/Plasmids	Relevant characteristics[a]	Reference or source
Strains		
E. faecalis		
OG1RF	Laboratory strain; BF^+; Ery^s, Fus^r, Kan^s, Rif^r	(5)
TX5421	OG1RF*ebpA*::pTEX5367; *ebpA* (ef1091) insertion disruption mutant of OG1RF; BF^-; Fus^r, Kan^r, Rif^r	This study
TX5460	OG1RF*ebpB*::pTEX5372; *ebpB* (ef1092) insertion disruption mutant of OG1RF; BF^-; Fus^r, Kan^r, Rif^r	This study
TX5448	OG1RF*ebpC*::pTEX5445; *ebpC* (ef1093) insertion disruption mutant of OG1RF; BF^-; Fus^r, Kan^r, Rif^r	This study
TX5470	OG1RFΔ*srtC*; *srtC* deletion mutant of OG1RF; BF^-; Fus^r, Rif^r	This study
TX5475	OG1RFΔ*ebpA*; *ebpA* allelic replacement *aph*(*3′*)-*IIIa* mutant of OG1RF; BF^-; Fus^r, Kan^r, Rif^r	This study
TX5482	TX5448 harboring plasmid pMSP3545; Kan^r, Ery^r (control for complementation)	This study
TX5476	TX5448 harboring plasmid pTEX5472; Kan^r, Ery^r (for complementation with the *ebpC* gene)	This study
TX5479	TX5448 harboring plasmid pTEX5478; Kan^r, Ery^r (for complementation with *ebpC* and *srtC* genes)	This study
TX5491	TX5470 harboring plasmid pMSP3545; Ery^r (control for complementation)	This study
TX5493	TX5470 harboring plasmid pTEX5478; Ery^r (for complementation with *ebpC* and *srtC* genes)	This study
TX5485	TX5475 harboring plasmid pMSP3545; Kan^r, Ery^r (control for complementation)	This study
TX5483	TX5475 harboring plasmid pTEX5472; Kan^r, Ery^r (for complementation with the *ebpC* gene)	This study

TX5484	TX5475 harboring plasmid pTEX5478; Kan^r, Ery^r (for complementation with *ebpC* and *srtC* genes)	This study
TX5494	TX5460 harboring plasmid pMSP3545; Kan^r, Ery^r (control for complementation)	This study
TX5495	TX5460 harboring plasmid pTEX5472; Kan^r, Ery^r (for complementation with the *ebpC* gene)	This study
TX5496	TX5460 harboring plasmid pTEX5478; Kan^r, Ery^r (for complementation with *ebpC* and *srtC* genes)	This study
E. coli		
DH5α	*E coli* host strain for routine cloning	Stratagene
TX5367	DH5α (pTEX5367); Kan^r	This study
TX5372	DH5α (pTEX5372); Kan^r	This study
TX5445	DH5α (pTEX5445); Kan^r	This study
TX5468	DH5α (pTEX5468); Kan^r	This study
TX5473	DH5α (pTEX5473); Kan^r, Ery^r	This study
TX5472	DH5α (pTEX5472); Kan^r, Ery^r	This study
TX5478	DH5α (pTEX5478); Kan^r, Ery^r	This study
Plasmids		
pTEX4577	Derived from pBluescript SK$^-$, used for insertion disruption mutagenesis in enterococci; Kan^r	(6)
pTEX4594	Suicide plasmid derived from pBluescript SK$^-$, used for mutagenesis in enterococci; Ery^r	This study
pMSP3545	Shuttle plasmid used for complementation, with nisin inducible promoter	(7)
pTEX5367	Intragenic *ebpA* fragment cloned into pTEX4577	This study
pTEX5372	Intragenic *ebpB* fragment cloned into pTEX4577	This study
pTEX5445	Intragenic *ebpC* fragment cloned into pTEX4577	This study

pTEX5468	Plasmid for *srtC* deletion; flanking regions of the *srtC* was cloned contiguously into pTEX4577	This study
pTEX5473	Plasmid for *ebpA* deletion; flanking regions of the *ebpA* and kanamycin resistance gene were cloned into pTEX4594	This study
pTEX5472	Construct for complementation; a 1986-bp fragment containing *ebpC* was cloned into the shuttle vector pMSP3545	This study
pTEX5478	Construct for complementation; a 3037-bp fragment containing *ebpC* and *srtC* was cloned into the shuttle vector pMSP3545	This study

[a]Fus, fusidic acid; Ery, erythromycin; Kan, kanamycin; Rif, rifampicin; Superscript "s" designates sensitivity, "r" designates resistance; for Kan "r" for enterococci indicates MIC >2000; BF^+, biofilm positive; BF^-, biofilm negative

Supplemental Table 2 Primers used in this study

Primer Name: sequence (5′→3′)[a]	Function Amplicon
Ef1091F: CCGCTCGAGAACTAACAAAAATGATTCGGCTCCAG Ef1091R: CCGCTCGAGCCATCTCACGCATTTTATCTTCAACT	Disruption mutant generation Intragenic *ebpA*
Ef1092F2: CCGCTCGAGCTGAAGGAAAAACGGTCCAA Ef1092R2: CCGCTCGAGCTTTTGCGTCGTCAGTGTGT	Disruption mutant generation Intragenic *ebpB*
Ef1093F: CCGGAATTCTGATAAATATCAAGGACTGGCAGA Ef1093R: CGGGGTACCTAAGCATACTCTCCAGAAGTCACG	Disruption mutant generation Intragenic *ebpC*
Ef1094Del_1F:AAAACTGCAGTCTGTAAATATTCCATTGGGGATT Ef1094Del_1R: TTTCGTCATATGCTTCCTCCTTAAAAATAAAGCA TGAGAG	Deletion mutant generation Upstream fragment of *srtC*
Ef1094Del_2F: GGAGGAAGCATATGACGAAAAGGCTAAACATA CTAAAAAA Ef1094Del_2R: TGCTCTAGAGTACGTTCGAGGTAATGCTAGGTT	Deletion mutant generation Downstream fragment of *srtC*
Ef1094delUpF: AACAATCAAACACCTGTTGAAAAA EF1094delDnR: TCGAAATCCCTATTTGATGCTTAT	Mutant confirmation
Ef1091Del_1F: CCGCTCGAGATTTGTTTCTAAAAGAGCGGAAA Ef1091Del_1R: CTATCACCTCGTCTGTTCTCTCCTTTCTTTTATGA ATTAA	Deletion mutant generation Upstream fragment of *ebpA*
mγδKanF: GAGAACAGACGAGGTGATAGGTAAGATTATACCGA GGTAT mγδKanR: TTTTCATTGAGCACGATGATCGTGCCGTGATCGAAA TCCA	Deletion mutant generation Kanamycin resistance gene
Ef1091Del_2F: ATCATCGTGCTCAATGAAAAACGCACGTTGGTTA AGTATT Ef1091Del_2R: TGCTCTAGATGCTTCATTTTCTCTCTCCTCTTT	Deletion mutant generation Downstream fragment of *ebpA*
Ef1091delUpF: AAGTCATTTCGACTTATGTCCT EF1091delDnR: AGCCTTCGCTTTTGGAAATAACAA	Mutant confirmation
Ef1091MupF: ATGAAATTTTACGTGCTAGGCAAT Ef1091MR: TATCCGTCTTTTTCAATTACAGCA	RT-PCR Upstream of *ebpA* to 5′ end of *ebpA*
Ef1091MupFa: AGAAATGAAACCGCTTGGTATG	RT-PCR Upstream of *ebpA*
Ef1091MF1: AATGTGTTAAACCATCAAGGGAAT Ef1091MR1: ACTCCTTTTTGAACTTCACCAATC	RT-PCR Intragenic *ebpA*
Ef1091MF2: TTAAAACAACGTGGGATTGAAATA Ef1091MR2: CACTTCTGGTAATGTTTGAACCTG	RT-PCR Intragenic *ebpA*
Ef1091MDownF: GGGGAAAAAGTAGCAGATGTTTTA Ef1092MR: AATTGACCATCAGGGAACAATAAT	RT-PCR 3′ end of *ebpA* to 5′ end of *ebpB*

Primer Name: sequence (5′→3′)[a]	**Function** **Amplicon**
Ef1092MF1: TAAATGGAGAAGATGGAGTGGTTT Ef1092MR1: ACAAACGGAATTCCTGATATCCTA	RT-PCR Intragenic *ebpB*
Ef1092MF2: CAAACAATGACTGTGTCGTATCAA Ef1092MR2a: AGAAGTACTGGCCATCTTTTAAAC	RT-PCR Intragenic *ebpB*
Ef1092MDownF: AGGCTTCTTACCAAAAACAAATGA Ef1093MR : TTCTTTCCCGCTATTTTGAATAAG	RT-PCR 3′ end of *ebpB* to 5′ end of *ebpC*
Ef1093MF1: ACAAGCTGTCCAAAGTTTAACTCC Ef1093MR1a : AGCCTTCGCTTTTGGAAATAACAA	RT-PCR Intragenic *ebpC*
Ef1093MF3: TTAAAAATGAGGCGAATGTTGATA Ef1093MR3 : CATCAGCCGTTGTTGTAAAAGTAG	RT-PCR Intragenic *ebpC*
Ef1093MDownFa: CAGTCTTGCTACTTATTGCAGGAG Ef1094MRa : CAAATGCACCTATTCCAATAATCA	RT-PCR 3′ end of *ebpC* to 5′ end of *srtC*
Ef1094MF2: AATGTCCGTTTACCAATTTTTGAT Ef1094MR2 : GGTGTGCAAGTTAATAAAGTGACG	RT-PCR Intragenic *srtC*
Ef1094MF5: TAAAAACCGTTGAACCAACTGATA Ef1094MR5 : GTACCAGATAATGAAGCCGCTAAT	RT-PCR Intragenic *srtC*
Ef1094MF: ATCGGTTATTAGTTCGAGGACATC Ef1094MDownR : AACTTGGCAGGAATTTAAACAGTC	RT-PCR 3′ end of *srtC* to downstream of *srtC*
gdhF: AGTGGCGCACTAAAAGATATGG gdhR : AGTTGTATTGAACCCTTGACCG	RT-PCR Intragenic *gdh*
Ef1093ComF2: AAAACTGCAGCAAAAAAGAGGAGAGAGAAAATG AAGCA Ef1093ComR2: TGCTCTAGATAAAAATAAAGCATGAAAGCCTGGG ACA	Complementation *ebpC*
Ef1093ComF: see above Ef1094ComR2: TGCTCTAGAAACTTGGCAGGAATTTAAACAGTC	Complementation *ebpC and srtC*
Ef1091F: see above Ef1091R: see above	Probe for colony hybridization *ebpA* probe
Ef1092F: CAGAAACCGGTGCAACAAATAGAAAAC Ef1092R: AAAAGACTTGCCGCCTGTACGAACTGT	Probe for colony hybridization *ebpB* probe
Ef1093F: see above Ef1093R: see above	Probe for colony hybridization *ebpC* probe

[a]Introduced restriction sites are underlined.

Supplemental data related References

1. Singh, K.V., Coque, T.M., Weinstock, G.M., and Murray, B.E. 1998. In vivo testing of an *Enterococcus faecalis efaA* mutant and use of *efaA* homologs for species identification. *FEMS Immunol Med Microbiol* 21:323-331.

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5. Murray, B.E., Singh, K.V., Ross, R.P., Heath, J.D., Dunny, G.M., and Weinstock, G.M. 1993. Generation of restriction map of *Enterococcus faecalis* OG1 and investigation of growth requirements and regions encoding biosynthetic function. *J Bacteriol* 175:5216-5223.

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10. Ton-That, H., and Schneewind, O. 2003. Assembly of pili on the surface of *Corynebacterium diphtheriae*. *Mol Microbiol* 50:1429-1438.

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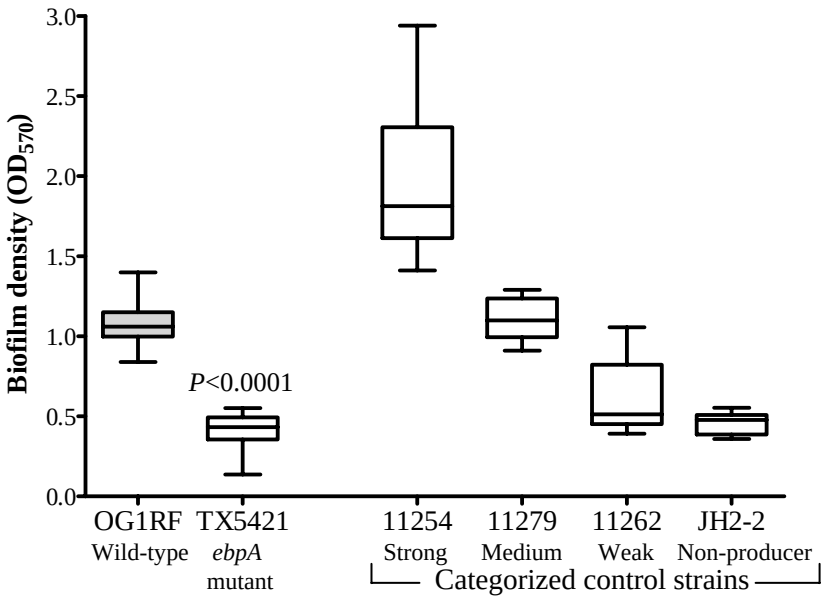
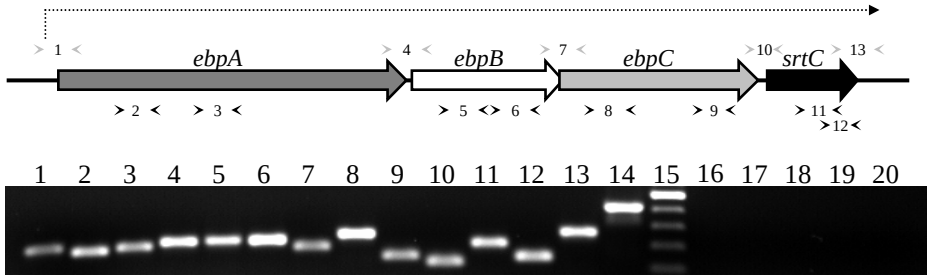


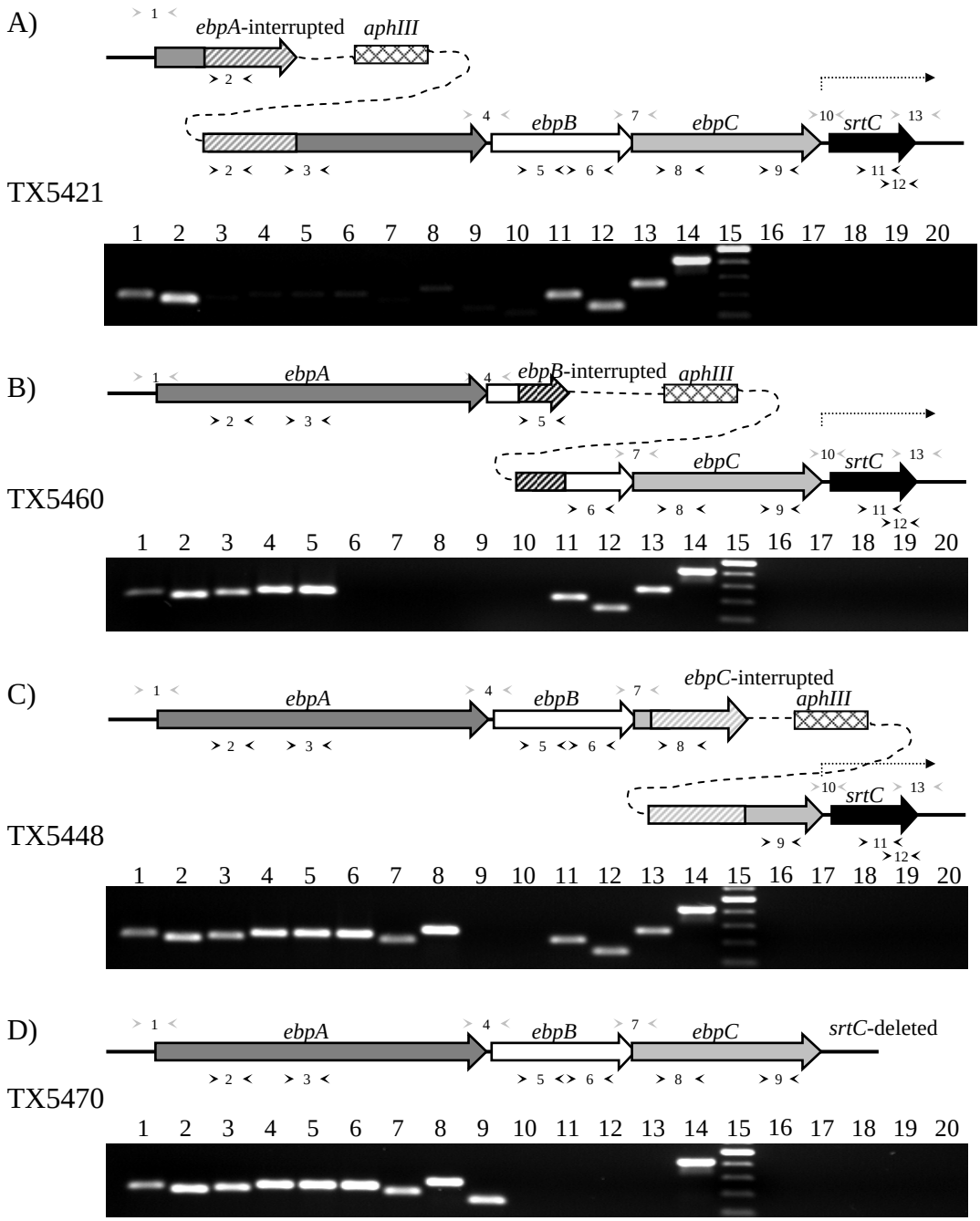
Supplemental Figure 1

Biofilm formation of OG1RF and the *ebpA* disruption mutant (TX5421). Median and interquartile range values are shown. Values are from at least three independent experiments, each performed in quadruplicate. Previous studies (8, 9) reported the median OD_{570} values for controls and these were >2 for *E. faecalis* strain 11254 (strong biofilm producer), 1 to 2 for strain 11279 (medium biofilm producer), 0.50 to < 1 for strain 11262 (weak biofilm producer), and ≤0.5 for strain JH2 (a non-biofilm producer).



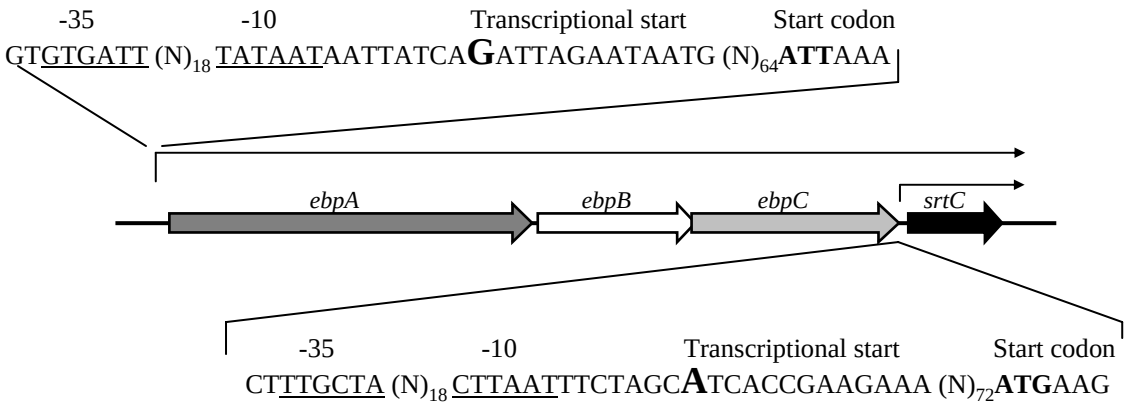
Supplemental Figure 2

RT-PCR analysis of the *ebpA,B,C* and *srtC* gene cluster using RNA extracted from cells of *E. faecalis* strain OG1RF (wild-type). Directions and locations of open reading frames of the genomic region analyzed are indicated by arrows and the transcript is indicated by the dotted line. Grey arrow heads marked on the top of the schematic show the locations and directions of primer sets used to amplify intergenic regions (see Supplemental Table 2). Primer set numbers positioned within arrow heads correspond to the lane numbers of the gel. Black arrow heads marked on the bottom of the schematic show the locations and directions of primers that amplified intragenic regions of *ebpA*, *ebpB*, *ebpC*, and *srtC* (lanes 2 and 3, lanes 5 and 6, lanes 8 and 9 and lanes 11 and 12, respectively). Results revealed that *ebpA* is co-transcribed with *ebpB* (335 bp product with primer set Ef1091MDownF and Ef1092MR, lane 4), that *ebpB* is co-transcribed with *ebpC* (306 bp product with primer set Ef1092MDownF and Ef1093MR, lane 7), and that *ebpC* is co-transcribed with *srtC* (254 bp product with primer set Ef1093MDownFa and Ef1094MRa, lane 10). A 528-bp fragment of the house-keeping gene *gdh* (lane 14) amplified using the gdhF and gdhR primers (Supplemental Table 2) was used as a positive control for RT reactions. 100 bp DNA ladder (Invitrogen) was used as the size marker (lane 15). For the samples in lanes 16 to 19 with intragenic primer sets 2, 5, 8, and 11, respectively, and in lane 20 with intragenic primers for *gdh*, the reverse transcriptase was omitted (control for DNA contamination).



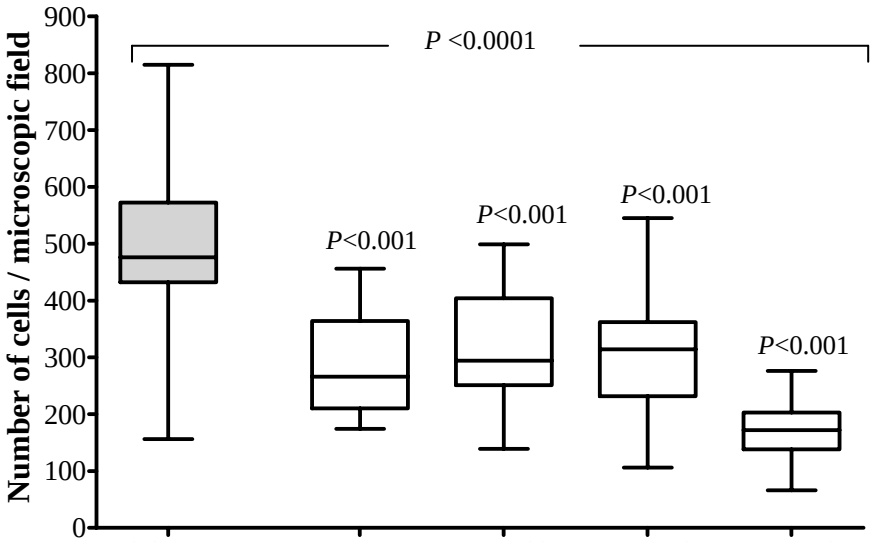
Supplemental Figure 3

RT-PCR analysis of RNA extracted from cells of *ebpA* (panel A), *ebpB* (panel B), *ebpC* (panel C) disruption mutants and *srtC* deletion mutant (panel D) of *E. faecalis* OG1RF with primer sets specified in the Supplemental Figure 2. Disruptions and deletion are schematically shown. Explanation for arrows and numbers is as described in the Supplemental Figure 2.



Supplemental Figure 4

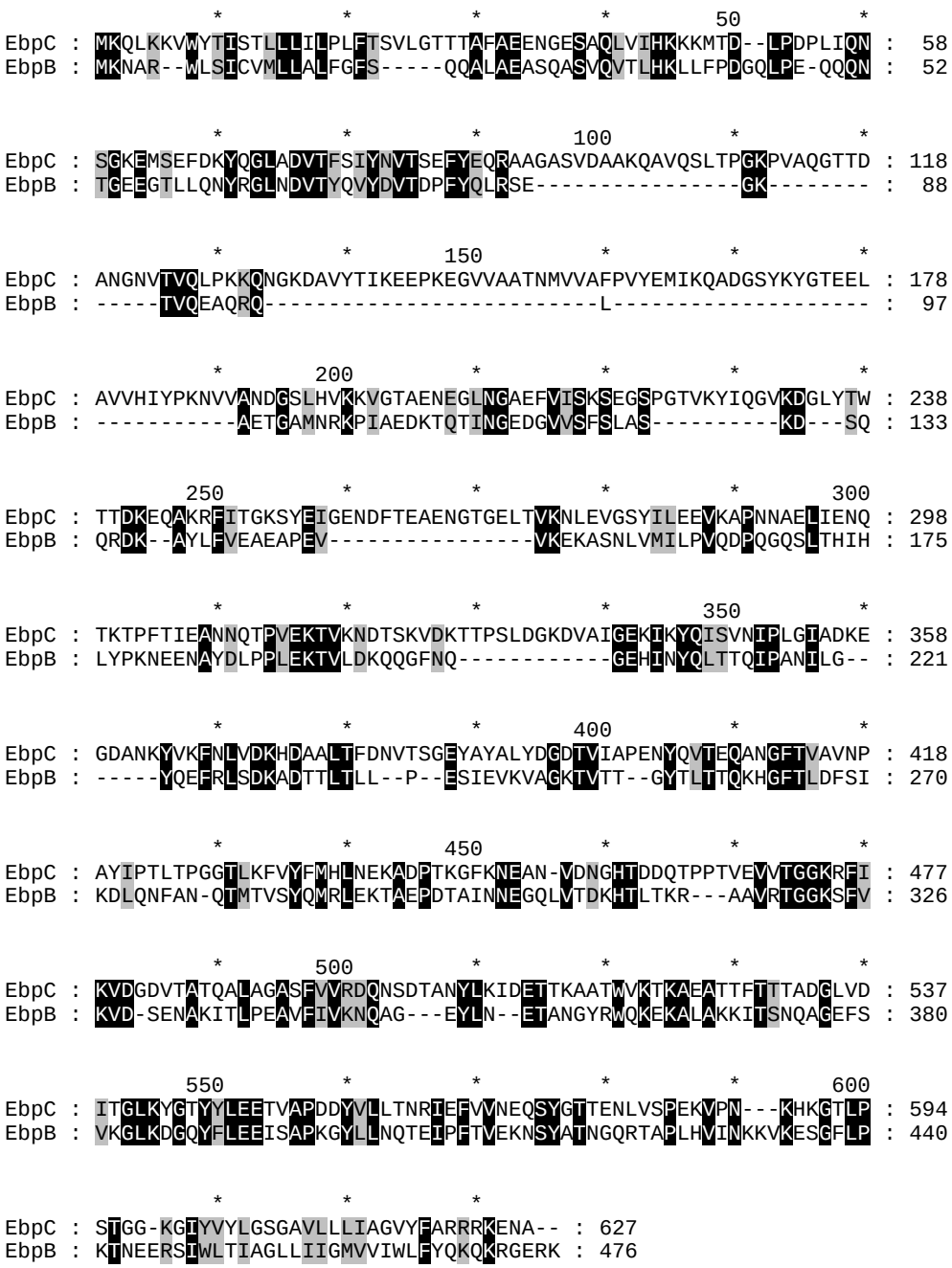
Promoter regions of the *ebpABC-srtC* operon and the *srtC* gene. Regions that contained the putative promoters and transcriptional and translational start sites of the 8694-bp DNA are shown. Start codons are shown in bold type. For the *ebpA* gene, no recognizable ribosome-binding site (RBS) is present in the immediate vicinity of the predicted start codon, ATG, of the V583 genome database annotation. Hence, the most likely start codon is ATT (positioned 9 bp downstream of a suitable RBS) which is located 120 bp upstream (the first 84 bp codes for a signal peptide) of the current genome database annotation predicted start codon. RT-PCR analysis that defined the limits *ebp* operon supports this prediction. The predicted transcriptional start site for each of the transcripts is marked with a bigger font letter and putative promoters are underlined. Predicted RBS, GGAG (preceding the start codons of *ebpA* by 9 bp, *ebpB* by 10 bp, and *ebpC* by 7 bp) and GGAGGAA (preceding the start codon of *srtC* by 3-bp) are not shown.



	OG1RF	TX5421	TX5460	TX5448	TX5470
	Wild-type	*ebpA* mutant	*ebpB* mutant	*ebpC* mutant	*srtC* mutant
Mean ± SD	497 ± 136	282 ± 85	322 ± 102	305 ± 114	173 ± 48

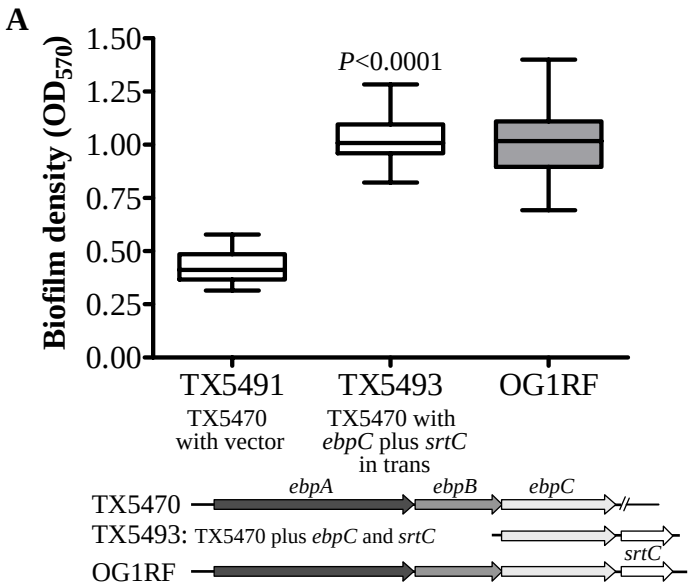
Supplemental Figure 5

Primary adherence assay with wild-type *E. faecalis* strain OG1RF and its isogenic derivatives with mutations introduced into *ebp* and *srtC* genes. Median and interquartile range values are shown. Adherent bacterial cells quantified using 25 fields of phase-contrast microscope represent three independent experiments. Multiple sample medians generated from the primary attachment quantifications were compared using Kruskal-Wallis analysis of variance (nonparametric analysis of variance), with Dunn's multiple comparison post-test. $P < 0.05$ was considered significant. Tests were performed by using GraphPad Prism v4 for Windows.



Supplemental Figure 6

Alignment of the complete amino acid sequences of EbpB and EbpC proteins. The alignment was performed using ClustalW (Lasergene software). Numbers on the top and at the right side of the alignment indicate the positions of the amino acid residues in the respective proteins. Identical residues are shaded in black and conservative substitutions are shaded in grey.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Biofilm density (OD570)
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1.25
1.00
0.75
0.50
0.25
0.00
P<0.0001
TX5491
TX5470 with vector
TX5493
TX5470 with ebpC plus srtC in trans
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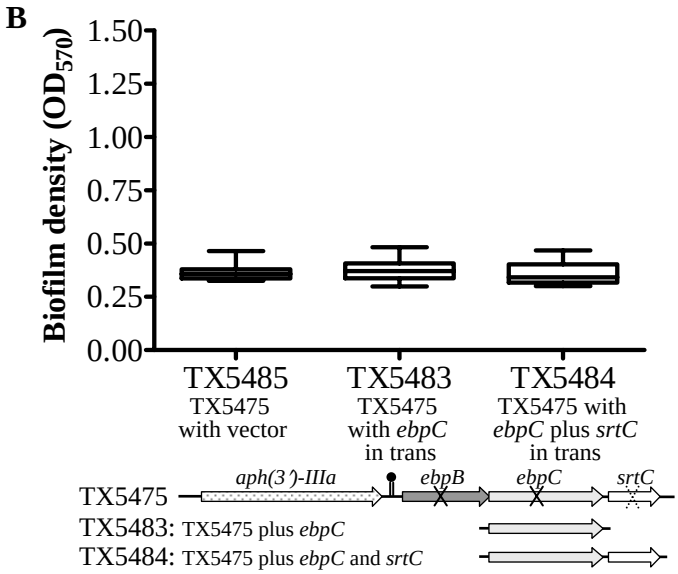
ebpB
ebpC
TX5470
TX5493: TX5470 plus ebpC and srtC
srtC
OG1RF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Biofilm density (OD570)
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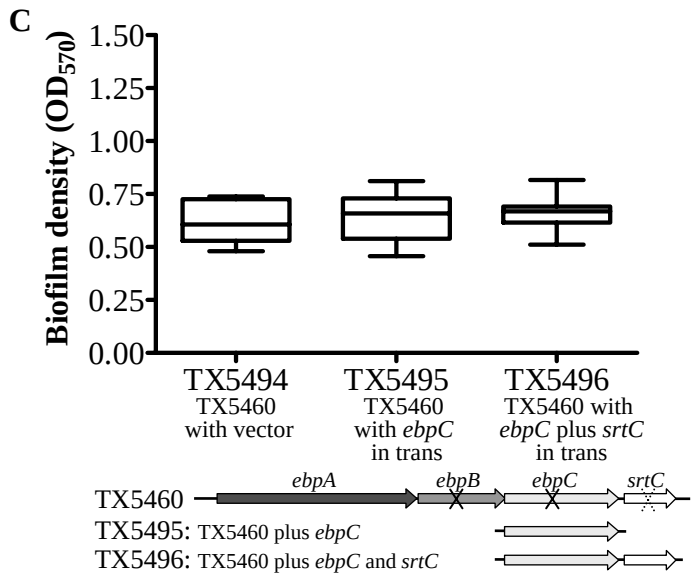
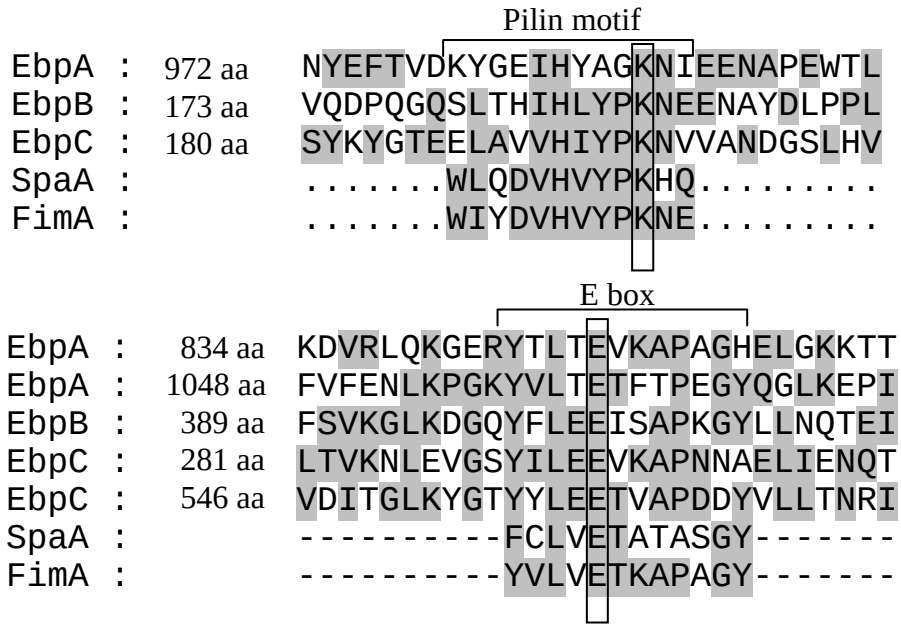
TX5485
TX5475 with vector
TX5483
TX5475 with ebpC in trans
TX5484
TX5475 with ebpC plus srtC in trans
aph(3')-IIIa
ebpB
ebpC
srtC
TX5475
TX5483: TX5475 plus ebpC
TX5484: TX5475 plus ebpC and srtC



Supplemental Figure 7

Complementation studies. Biofilm formation after complementing A) the *srtC* deletion mutant with *ebp*C plus *srt*C cloned under control of the nisin inducible promoter of pMSP3545 (TX5493), B) *ebp*A deletion mutant with *ebp*C or *ebp*C plus *srt*C (TX5483 and TX5484, respectively), and C) the *ebp*B mutant with *ebp*C or *ebp*C plus *srt*C (TX5495 and TX5496, respectively). Median and interquartile range values are shown. Values are from at least three independent experiments, each performed in quadruplicate. The schematic below each panel illustrates the gene disruptions, deletions, and complementation fragments. Inactive ebp genes are cross marked with a solid line. Since the *srt*C gene is on two different transcripts, reduced transcription due to loss of *ebp-srt*C transcript is cross marked with dashed line. OD_{570} medians from biofilm assays of complemented strains were compared with appropriate vector alone control strains using Mann-Whitney test and $P < 0.05$ was considered significant. Tests were performed by using GraphPad Prism v4 for Windows.



Supplemental Figure 8

Conserved lysine (K) and glutamic acid (E) residues in pilin motif and E-box of *E. faecalis* Ebp proteins. Motifs common to all three Ebp proteins were identified with Genetics Computer Group (GCG) software package. Base position with respective to the start codon is shown for each motif. Similar motifs (pilin motif, WxxxVxVY**PK**N; E-box, Yx**L**x**ET**xAPxGY) were previously characterized in pilus-forming Spa proteins of *C. diphtheriae* and fimbriae forming Fim proteins of *Actinomyces naeslundii* (10, 11) and are shown here.