Supplemental material

Contents:

TABLE S1 Plasmids and strains used in this study

Name	Description[a]	Source
Vectors		
pLT06	Vector for unmarked gene deletions in *E. faecalis*; cm^r	(1)
pTCV-*lac*	*E. coli*-Gram positive shuttle vector for transcriptional fusions to *lacZ*; erm^r, kan^r	(2)
pAT28	*E. coli*-Gram positive shuttle vector; spc^r	(3)
pAH328	Vector for transcriptional promoter fusions to *luxABCDE* (luciferase) in *B. subtilis*; integrates in *sacA*; cm^r	(4)
pBS2E	Empty vector for *B. subtilis* for cloning in the BioBrick standard; integrates in *lacA*; mls^r	(5)
pXT	Vector for xylose-inducible gene expression in *B. subtilis*; integrates in *thrC*; spc^r	(6)
pSB1A3	*E. coli* vector for cloning in the BioBrick standard; amp^r	RSBP[b]
Plasmids		
pAT2050-49	pAT28 containing the EF2050-2049 operon with its native promoter region	This study
pLT0927	pLT06 containing the deletion construct for EF0927	This study
pLT2050-49	pLT06 containing the deletion construct for EF2050-2049	This study
pLT2752-51	pLT06 containing the deletion construct for EF2752-2751	This study
pTCV56	pTCVlac containing a transcriptional P_{EF2050}-*lacZ* fusion from -113 to +52 relative to the start codon	This study
pTCV57	pTCVlac containing a transcriptional P_{EF2050}-*lacZ* fusion from -87 to +52 relative to the start codon	This study
pTCV58	pTCVlac containing a transcriptional P_{EF2050}-*lacZ* fusion from -77 to +52 relative to the start codon	This study
pTCV59	pTCVlac containing a transcriptional P_{EF2752}-*lacZ* fusion from -106 to +49 relative to the start codon	This study
pTCV60	pTCVlac containing a transcriptional P_{EF2752}-*lacZ* fusion from -67 to +49 relative to the start codon	This study
pTCV0926	pTCVlac containing a transcriptional P_{EF0926}-*lacZ* fusion from -233 to +59 relative to the start codon	This study
pCF129	pXT containing the EF2752-2751 operon	This study
pCF130	pXT containing the EF2050-2049 operon	This study
pCF134	pAH328 containing a transcriptional P_{EF2050}-*luxABCDE* fusion from -190 to +94 relative to the start codon	This study
pCF135	pAH328 containing a transcriptional P_{EF2752}-*luxABCDE* fusion from -180 to +51 relative to the start codon	This study
pCF143	pSB1A3 containing the EF0926-0927 operon in the BioBrick format	This study
pCF144	pSB1A3 containing *B. subtilis* P_{bceR} region from -287 to -29 relative to the start codon in the BioBrick format	This study
pCF145	pBS2E containing the EF0926-0927 operon under control of *B. subtilis* P_{bceR}	This study
pSG704	pXT containing *B. subtilis bceAB*	(7)
E. coli		
DH5α	*supE44* Δ*lacU169(*Φ*80lacZ*Δ*M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	(8)
MC1061	F^- Δ(*ara-leu*)7697 [*araD139*]$_{B/r}$ Δ(*codB-lacI*)3 *galK16 galE15* λ^- $e14^-$ *mcrA0 relA1 rpsL150*(*strR*) *spoT1 mcrB1 hsdR2*(r^-m^+)	(9)
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac F′::*Tn*10 proAB lacI*^{q} Δ(*lacZ*)M15]	Stratagene
E. faecalis		
JH2-2	Laboratory strain, plasmid-free; rif^r, fs^r	(10)
V583	Sequenced clinical strain containing plasmids pTEF1, pTEF2, pTEF3; van^r	(11)
Δ0927	JH2-2 carrying an unmarked deletion of EF0927	This study
Δ2050-49	JH2-2 carrying an unmarked deletion of the EF2050-2049 operon	This study
Δ2752-51	JH2-2 carrying an unmarked deletion of the EF2752-2751 operon	This study
Δ2050-49 Δ2752-51	JH2-2 carrying unmarked deletions of the EF2050-2049 and EF2752-51 operons	This study
DLEf01	JH2-2 carrying pTCV56; kan^r	This study
DLEf02	JH2-2 carrying pTCV 57; kan^r	This study
DLEf03	JH2-2 carrying pTCV 58; kan^r	This study
DLEf04	JH2-2 carrying pTCV 59; kan^r	This study
DLEf05	JH2-2 carrying pTCV 60; kan^r	This study

DLEf06	JH2-2 carrying pTCV0926; kan^r	This study
DLEf07	Δ0927 carrying pTCV56; kan^r	This study
DLEf08	Δ0927 carrying pTCV59; kan^r	This study
DLEf09	Δ0927 carrying pTCV0926; kan^r	This study
DLEf10	Δ2050-49 carrying pTCV56; kan^r	This study
DLEf11	Δ2050-49 carrying pTCV59; kan^r	This study
DLEf12	Δ2050-49 carrying pTCV0926; kan^r	This study
DLEf13	Δ2752-51 carrying pTCV56; kan^r	This study
DLEf14	Δ2752-51 carrying pTCV59; kan^r	This study
DLEf15	Δ2752-51 carrying pTCV0926; kan^r	This study
DLEf16	Δ2050-49 carrying pAT2050-49; spc^r	This study
B. subtilis		
W168	Wild-type, *trpC2*	Laboratory stock
SGB220	TMB1518 *lacA*::pCF145 *sacA*::pCF134; cm^r, mls^r	This study
SGB221	TMB1518 *lacA*::pCF145 *sacA*::pCF135; cm^r, mls^r	This study
SGB222	TMB1518 *lacA*::pCF145 *thrC*::pCF129 *sacA*::pCF134; cm^r, mls^r, spc^r	This study
SGB223	TMB1518 *lacA*::pCF145 *thrC*::pCF129 *sacA*::pCF135; cm^r, mls^r, spc^r	This study
SGB224	TMB1518 *lacA*::pCF145 *thrC*::pCF130 *sacA*::pCF134; cm^r, mls^r, spc^r	This study
SGB225	TMB1518 *lacA*::pCF145 *thrC*::pCF130 *sacA*::pCF135; cm^r, mls^r, spc^r	This study
SGB228	TMB1518 *sacA*::pCF134; cm^r	This study
SGB229	TMB1518 *sacA*::pCF135; cm^r	This study
SGB231	TMB1518 *thrC*::pCF129; spc^r	This study
SGB233	TMB1518 *thrC*::pCF130; spc^r	This study
TMB1518	W168 with unmarked deletions of the *bceRS-bceAB*, *psdRS-psdAB*, *yxdJK-yxdLM-yxeA* loci	Own unpublished study
TMB1555	TMB1518 *thrC*:: pSG704; spc^r	This study

[a] Amp, ampicillin; cm, chloramphenicol; erm, erythromycin; fs, fusidic acid; kan, kanamycin; mls, macrolide-lincosamide-streptogramin B group; rif, rifampin; spc, spectinomycin; van, vancomycin; r, resistant

[b] RSBP, Registry of Standard Biological Parts (http://parts.igem.org)

TABLE S2 Primers used in this study

Primer name	Sequence (5'-3') [a]	Use
Oligonucleotides for cloning *E. faecalis* constructs		
DLEF_034	ATAGGATCCGTGGGATTGCATGGCTAAAATCAT	EF0927 deletion up fwd
DLEF_035	gacctactttTGTCTACTCCTATCTAGGGCACCATAT	EF0927 deletion up rev
DLEF_036	ggagtagacaAAAGTAGGTCTATCAGCAAACTAAAGAGG	EF0927 deletion down fwd
DLEF_037	CGCCTGCAGATACCCTAAAAGAATACCACCTA	EF0927 deletion down rev
DLEF_017	AGTGGATCCTAGGAACGTTTTTACCAAC	EF2050-2049 deletion up fwd
DLEF_031	ttttttataattaatctAACATATTTTCCACTCCTATTCTTCTCACTC	EF2050-2049 deletion up rev
DLEF_030	gaaaatatgttAGATTAATTATAAAAAATCGTTAAGCATGC	EF2050-2049 deletion down fwd
DLEF_020	GGAACTGCAGTAACATAAGAAAGTTTTTTCT	EF2050-2049 deletion down rev
DLEF_022	GCCGGATCCATCAATTAAACAGCAGTTT	EF2752-2751 deletion up fwd
DLEF_032	tacagttttttaaTTTTCTCGCTCCAACGTTTTGTT	EF2752-2751 deletion up rev
DLEF_033	gagcgagaaaaTTAAAAAACTGTAAAAGCCGTTGTGAA	EF2752-2751 deletion down fwd
DLEF_025	CATCTGCAGTAATTCTTCCGCCGTA	EF2752-2751 deletion down rev
DLEF_056	GCATGAATTCTAAAAGAATAACAGTGCACTTACAGTAA	pTCV56 fwd
DLEF_057	GCACGAATTCTAATGTAAGTTCATTGTTATTCTTTTCTATTGTCG	pTCV57 fwd
DLEF_058	TAATGGAATTCCATTGTTATTCTTTTCTATTGTCGTTTTTTTTC	pTCV58 fwd
DLEF_008	TTAAGGATCCATATTTAATTTCGTTTCCGTAAAC	P_{EF2050} rev
DLEF_059	TTCAGAATTCGCCAATTATTCTTTGACTACCGAACGATAT	pTCV59 fwd
DLEF_060	TGTGAATTCAAACGATGTTCATTCAGATTTTTTCTTGGTAAAA	pTCV60 fwd
DLEF_010	TTATGGATCCAACGACCATATGTCTTGCTGA	P_{EF2752} rev
DLEF_052	AAAAGAATTCGTCCATGCCAATTGTCGAAAAAGCTTCTATT	pTCV0926 fwd
DLEF_053	TTTTGGATCCTCCTCGCTAATTAATTCTCGAATGGTTGT	pTCV0926 rev
DLEF_061	ATTTTGGATCCTTAATCTTTCACTAAGTGATAAACTTTTTTGA	pAT-2050-49 rev
Oligonucleotides for 5'-RACE		
DLEF_046	GTAACAAGCCCATCACATCCTTAGATGA	EF2050 5'-RACE-1
DLEF_047	TGCTTGTCCACCAGAAATTTCT	EF2050 5'-RACE-2
DLEF_048	CATTACAGAGACTTTTTCTCCATCAAGTGTCAATGGC	EF2050 5'-RACE-3
DLEF_049	ACAATTCTGTTGCTGATTTTGAATCCAG	EF2752 5'-RACE-1
DLEF_050	CCCACAGATATCTCTGGTGGATAA	EF2752 5'-RACE-2
DLEF_051	CTTCTAATGAAACTTTTTCTACAGCTAAAGGCAACAGAATATTGT	EF2752 5'-RACE-3
Oligonucleotides for cloning *B. subtilis* constructs		
TM2445	AAATTGGATCCAATAACAAAACGTTGGAGCG	pCF129 fwd
TM2446	AAATTGAATTCTTCACAACGGCTTTTACAG	pCF129 rev
TM2447	AAATTGGATCCCGATACACTAAATGAGTGAG	pCF130 fwd
TM2448	AAATTGAATTCAAAATCGTTAAGCGTGCATG	pCF130 rev
TM2596	AAATTGAATTCGCCAAATTGCTAAAAGCAAC	pCF135 fwd
TM2597	AAATTACTAGTTAAACGACCATATGTCTTGC	pCF135 rev
TM2598	AAATTGAATTCTCGTTGCGTAGAATATTCAG	pCF134 fwd
TM2599	AAATTACTAGTAAATTCACCATCTTGGACAG	pCF134 rev
	AAATTGAATTC**GCGGCCGC**tTCTAGA**AAGGAGG**tgGCCGGC**ATG**GCT	pCF143 fwd
TM2600	AAAATCATGATTGTCGAAG	
TM2601	AAATTACTAGTA**TTA**ACCGGTTTTCACTTCATTATAATAACTGAGGG	pCF143 rev
TM2610	AATTGAATTCGCGGCCGCTTCTAGAGTTCCGATGATTCTTGCGGCC	pCF144 fwd
TM2611	AATTACTAGTACAAGTGTATAGCAAAACGCC	pCF144 rev

[a] Restriction sites are underlined; overlaps to other primers for PCR fusions are shown by lower case letters; the optimised Shine-Dalgarno sequence and start and stop codons for the EF0926-0927 BioBrick are in bold

Supplemental Text: Detailed description of the proteomic analysis

Methods

For proteomic analyses, exponentially growing cultures (OD_{600} = 0.4) of *E. faecalis* V583 in BHI medium were exposed to 64 µg ml^{-1} bacitracin for 1 h. Control cells were treated with an equal volume of sterile water. Cells were harvested by centrifugation, washed twice with 10 mM Tris/HCl (pH 7) and stored at -80°C.

For protein extraction, cells from 0.4 L culture volume were resuspended in 5 ml lysis buffer (20 mM Tris, 7.4 M urea, 2 M thiourea, 3 % CHAPS, 1 % Brij-35, 1 mM PMSF, 40 mM DTT, 5 mM TBP, 0.5 % (v/v) carrier ampholyte 3-10 (Invitrogen)). Cells were disrupted by four passages through a French pressure cell at 20,000 psi, followed by six 15 s cycles of sonication (Vibra-Cell SONICS) at 20 % amplitude. The soluble fraction was isolated by centrifugation for 5 min at 25,900 × *g*, followed by 45 min at 186,000 × *g*, and stored at -80°C. Protein concentrations were determined with a 2-D Quant kit (GE Healthcare), and 300 µg of protein were then treated with the 2-D Clean up kit (GE Healthcare) according to the manufacturer's instructions.

For isoelectric focussing, samples were mixed with rehydration buffer (7 M urea, 2 M thiourea, 2 % CHAPS, 1 % Brij-35, 50 mM DTT, 4 mM TCEP, 0.5 % (v/v) carrier ampholytes pH 3-10 (Invitrogen), 5 mM TBP; total loading volume 315 µl) and loaded onto an 18 cm pH 4-7 ReadyStrip™ IPG strip (Bio-Rad) by passive rehydration for 16 hours at room temperature. Subsequently, isoelectric focussing was performed with an IPGphor Isoelectric Focussing unit (GE Healthcare) using a step-wise protocol of 2 h at 200 V, 3 h at 500 V, 4 h at 1000 V, 3 h at 2000 V, 6 h at 4000 V and 7 h at 8000 V.

IPG strips were incubated for 10 min in 1.7 ml reduction solution (6 M urea, 130 mM DTT, 375 mM Tris-HCl, pH 8.8, 0.1 % (w/v) SDS, 20% glycerol, 2% (v/v) saturated bromophenol blue solution), followed by 10 min in 1.7 ml alkylation solution (same as reduction solution but containing 135 mM iodoacetamide instead of DTT). Second dimension SDS-PAGE was carried out using 12.5 % acrylamide gels in the Laemmli buffer system (12) and using an Ettan™ DALT*six* Electrophoresis System (GE Healthcare) according to the manufacturer's instructions.

Gels were fixed twice in 10% methanol, 7% acetic acid for 30 min, stained overnight with SYPRO-ruby (Invitrogen) and destained twice in distilled water for 30 min. Image acquisition was carried out using a Molecular Imager FX (Bio-Rad) at a resolution of 100 microns, and image analysis was performed using the PDQuest 2-D analysis software (Bio-Rad) according to the manufacturer's recommendations. Spots of significantly different intensity, based on two-tailed paired t-test analysis, were excised subsequently from colloidal Coomassie-stained gels (13), digested in-gel with trypsin and peptides identified using a 4800 MALDI TOF/TOF Analyzer (Applied Biosystems). The acquired spectra from the MALDI-TOF/TOF were analysed with MASCOT (Matrix Science) against the NCBInr database. MASCOT matches were only accepted with a probability score of $p < 0.05$. Proteins with one peptide matched were manually confirmed by the Centre for Protein Research, University of Otago.

Results

To investigate the response of *E. faecalis* to bacitracin, we analysed the proteome of strain V583, whose genome has been fully sequenced, after 1 h exposure to 64 μg ml^{-1} bacitracin, which corresponded to the strain's minimal inhibitory concentration (MIC), compared to an untreated control. A summary of the differentially produced proteins is given in Table 1; the 2D-gels and detailed analysis of protein spots are available as supplementary files Figure S1 and Table S3, respectively. As expected, a number of proteins involved in cell wall metabolism were found in increased quantities after bacitracin stress. These included enzymes involved in rhamnose biosynthesis (EF2191, EF2192, EF2193), a major component of polysaccharides in the enterococcal cell wall (14). Furthermore, increased production was observed for EF2172, proposed to be involved in isoprenoid biosynthesis, which is required for production of the lipid carrier molecule undecaprenyl-(pyro)phosphate (15), the cellular target of bacitracin (16). Additionally, several enzymes for energy metabolism (pyruvate dehydrogenase, 6-phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase) were found up-regulated, while protein synthesis (ribosomal proteins S6, L31 and L7/L12) appeared down-regulated, indicating metabolic re-arrangements in response to bacitracin. This is also seen for fatty acid metabolism with up-regulation of one isoform of 3-oxoacyl-ACP synthase (FabF-1) and down-regulation of another (FabF-2). Further proteins with increased production after bacitracin exposure likely indicated a general response to stress, including enzymes involved in nucleotide salvage (EF0264, EF2549, EF2694) and correction of protein misfolding (EF0266, EF0771, EF1647).

While most of the proteins discussed above showed changes in the range of two- to six-fold, only three spots displayed larger changes. The strongest response, over 50-fold, was observed for the hypothetical protein encoded by EF1753. This protein contains the domain COG3595 and is thus similar to YvlB of *B. subtilis*. While the cellular role of YvlB is still unknown, its gene is part of the cell wall stress stimulon of *B. subtilis*, which is induced, for example, by bacitracin (17, 18). It therefore appears likely that YvlB and EF1753 share a similar function. The second most highly regulated protein was EF2586, annotated as encoding cobyric acid synthase. These enzymes are involved in vitamin B12 biosynthesis (19), but the reason for its strong response to bacitracin is unclear. The third strongest effect was observed for EF2050. This protein is the ATPase component of a BceAB-like ABC-transporter (EF2050-2049), which have to date only been identified in the context of resistance against peptide antibiotics (20, 21). As mentioned above, two such transporters had been previously identified in the genome of *E. faecalis* by a comparative genomics study, namely EF2050-2049 and EF2752-2751 (20). Although our proteomic study only identified the former transporter, both loci were found to be up-regulated in response to bacitracin by transcriptome analysis (22). We therefore decided to investigate these two transporters in more detail, both regarding their role in bacitracin resistance and their regulation.

Discussion

Our proteomic analysis of bacitracin-exposed *E. faecalis* showed that the bacterium responds to the antibiotic with a specific reaction to the bacitracin-induced cell wall damage, as well as with a more general response to stress or growth inhibition. Cell wall damage is counteracted

for example by increased synthesis of cell wall polysaccharides as seen by the up-regulation of the rhamnose biosynthetic pathway. Changes in the cell surface have been implicated in resistance against antimicrobial peptides, either by altering the surface charge or decreasing access of the peptide to its cellular target (23, 24), consistent with our findings. One well-characterized example for such cell wall modifications is the D-alanylation of teichoic acids, catalyzed by the Dlt-system (25, 26), which reduces the net negative charge of the cell surface and thus the affinity of the primarily cationic peptides. While the entire *dltABCD*-operon of *E. faecalis* OG1RF (EF2750-EF2746) was found up-regulated in response to bacitracin and vancomycin by transcriptome analysis (22), none of the corresponding proteins was identified in our proteomic approach. This may be due to the membrane-localization of at least two of the Dlt-proteins (27), which would have excluded them from the 2D-PAGE analysis.

Most of the proteins with differential production after bacitracin stress had metabolic functions and thus are more likely to represent a general response to stress rather than a specific reaction to bacitracin. Abranches and colleagues reported an apparent metabolic down-shift after bacitracin-stress, as deduced from their transcriptome study (22). However, our data seem more consistent with metabolic re-arrangements. In good agreement with the previous study, we also observed a decrease in protein synthesis exemplified by the down-regulation of several ribosomal proteins. In contrast, a number of enzymes of energy metabolism, particularly of glycolysis, were found up-regulated in our study, possibly reflecting an increased demand for energy to combat the stress condition. It is not reasonable to expect identical results from transcriptomic and proteomic analyses of the same condition, because transcriptomics cannot detect post-transcriptional regulation, while proteome analyses exclude hydrophobic proteins or others that are insoluble under assay conditions. With this in mind, our data are overall consistent with those reported previously, and together these two datasets provide a useful overview of the response of *E. faecalis* to inhibitors of cell wall synthesis such as bacitracin.

TABLE S3 Summary of differentially produced proteins after bacitracin stress in *E. faecalis* V583

Locus tag	Gene	Spot[a]	Annotation	x-fold change[a,b]	p-value
Up-regulated proteins					
EF0233	*rpoA*	24	DNA-directed RNA polymerase subunit alpha	2.3	0.044
EF0264	*hpt*	35	Hypoxanthine-guanine phosphoribosyltransferase	5.7	0.027
EF0266		47	Chaperonin, 33 kDa	2.2	0.039
EF0283	*fabF-1*	30	3-oxoacyl-ACP synthase	2.6	0.061
EF0445	*menB*	40	Naphthoate synthase	5.3	<0.001
EF0771	*clpP*	31	ATP-dependent Clp protease proteolytic subunit	2.9	<0.001
EF0912		19	Peptide ABC transporter ATP-binding protein	2.6	0.024
EF1045	*pfk*	21	6-phosphofructokinase	2.2	0.067
EF1142		48	HAD superfamily hydrolase	2.1	0.05
EF1353	*pdhA*	18	Pyruvate dehydrogenase complex E1 component subunit alpha	1.9	0.022
EF1354	*pdhB*	22	Pyruvate dehydrogenase complex, E1 component subunit beta	2.4	0.014
EF1356	*lpdA*	26	Dihydrolipoamide dehydrogenase	2.1	0.015
EF1402		49	Hypothetical protein	2.5	0.022
EF1586	*nox*	23	NADH oxidase	1.9	0.023
EF1647	*hslV*	41	ATP-dependent protease peptidase subunit	6.9	0.007
EF1746	*galU*	27	UTP-glucose-1-phosphate uridylyltransferase	2.8	0.1
EF1753		42/44	Hypothetical protein (COG3595)	55.9/33.3	0.019/ 0.002
EF1964	*gap-2*	16/17	Glyceraldehyde-3-phosphate dehydrogenase	2.4/1.8	0.099/ 0.106
EF2050		43	ABC transporter ATP-binding protein	9.6	0.001
EF2172	*ispD*	15	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase	1.9	0.022
EF2191		38	dTDP-4-dehydrorhamnose reductase	6.0	0.001
EF2192	*rfbB*	20	dTDP-glucose 4,6-dehydratase	2.5	0.067
EF2193	*rfbC*	37	dTDP-4-dehydrorhamnose 3,5-epimerase	5.4	0.032
EF2421	*thrC*	50	Threonine synthase	2.6	0.019
EF2432		51	Metallo-beta-lactamase superfamily protein	3.6	0.016
EF2549	*upp*	36	Uracil phosphoribosyltransferase	3.3	0.029
EF2586		52	Cobyric acid synthase	15.2	0.017
EF2694	*pfs*	33	MTA/SAH nucleosidase	5.1	0.053
EF2729	*nusG*	29	Transcription antitermination protein	2.9	0.02
EF2879	*accB*	28	Acetyl-CoA carboxylase	2.2	<0.001
EF2954		53	Cro/CI family transcriptional regulator	4.0	0.019
Down-regulated proteins					
EF0007	*rpsF*	5	30S ribosomal protein S6	0.3	0.018
EF0185	*deoB*	9	Phosphopentomutase	0.4	0.09
EF0709	*ptsH*	6	Phosphocarrier protein HPr	0.3	0.028
EF0760		12	Amino acid ABC transporter ATP-binding protein	0.6	0.058
EF1171	*rpmE*	7	50S ribosomal protein L31	0.3	0.053
EF2556		4	Fumarate reductase flavoprotein subunit	0.1	0.076
EF2715	*rplL*	8	50S ribosomal protein L7/L12	0.2	0.082
EF2880	*fabF-2*	14	3-oxoacyl-ACP synthase	0.6	0.034
EF2923		2	Hypothetical protein EF2923	0.2	0.012
EF3118	*rpe*	10	Ribulose-phosphate 3-epimerase	0.5	0.033
EF3256		13	Pheromone cAD1 lipoprotein	0.6	0.09

[a] Spot numbers refer to the markings in supplementary Figure S1; where a single protein was identified in two spots, both spot numbers and both induction values are given.

[b] Represents the x-fold change in spot intensity in the bacitracin-stressed samples relative to the control; details on quantification and statistical analyses are given in Table S3.

TABLE S4 Analysis of differentially produced proteins from 2D-PAGE of bacitracin-stressed and unstressed samples

	PDQuest and Excel analysis				MALDI-TOF/TOF (MS/MS) and MASCOT analysis					
Spot number [a]	Average control spot quantity [b]	Average bacitracin stress spot quantity [c]	p value [d]	x-fold change [e]	Protein identified	Accession number (MASCOT)	MASCOT Score	Number of peptides matched [f]	Nominal mass	Calculated pI
Up-regulated proteins										
15	695.2	1346.3	0.022	1.9	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase	gi | 29376680	248	3	26847	5.4
16	1187.8	2826.5	0.099	2.4	Glyceraldehyde-3-phosphate dehydrogenase	gi | 29376486	670	5	35749	5.0
17	9399.2	16682.9	0.106	1.8	Glyceraldehyde-3-phosphate dehydrogenase	gi | 29376486	1086	9	35749	5.0
18	1707.8	3315.0	0.022	1.9	Pyruvate dehydrogenase complex E1 component subunit alpha	gi | 29375920	664	6	41358	5.3
19	1005.9	2606.3	0.024	2.6	Peptide ABC transporter ATP-binding protein	gi | 29375496	643	6	35768	5.9
20	1412.6	3572.0	0.067	2.5	dTDP-glucose 4,6-dehydratase	gi | 29376700	962	9	38315	4.9
21	2314.6	5126.0	0.067	2.2	6-phosphofructokinase	gi | 29375624	596	6	34391	5.6
22	1147.3	2796.8	0.014	2.4	Pyruvate dehydrogenase complex, E1 component subunit beta	gi | 29375921	806	8	35373	4.7
23	978.8	1873.5	0.023	1.9	NADH oxidase	gi | 29376148	862	8	48884	4.8
24	908.4	2078.5	0.044	2.3	DNA-directed RNA polymerase subunit alpha	gi | 29374877	457	4	35028	4.9
26	865.7	1779.5	0.015	2.1	Dihydrolipoamide dehydrogenase	gi | 29375923	916	6	49113	4.9
27	840.4	2392.8	0.100	2.8	UTP-glucose-1-phosphate uridylyltransferase	gi | 29376295	641	7	33460	5.4
28	976.7	2111.1	<0.001	2.2	Acetyl-CoA carboxylase	gi | 29377345	112	1	17549	4.2
29	909.2	2596.6	0.020	2.9	Transcription antitermination protein NusG	gi | 29377206	434	3	20413	4.8
30	651.8	1726.7	0.061	2.6	3-oxoacyl-ACP synthase	gi | 29374922	269	3	43169	5.1
31	2124.2	6150.5	<0.001	2.9	ATP-dependent Clp protease proteolytic subunit	gi | 29375364	420	4	21608	4.6
33	322.2	1646.2	0.053	5.1	MTA/SAH nucleosidase	gi | 29377174	593	5	24633	4.9
35	520.3	2949.0	0.027	5.7	Hypoxanthine-guanine phosphoribosyltransferase	gi | 29374905	616	5	20260	4.7
36	834.6	2790.7	0.029	3.3	Uracil phosphoribosyltransferase	gi | 29377038	367	4	22870	5.3
37	485.7	2630.4	0.032	5.4	dTDP-4-dehydrorhamnose 3,5 - epimerase	gi | 29376701	540	5	21323	5.4
38	352.7	2128.1	0.001	6.0	dTDP-4-dehydrorhamnose reductase	gi | 29376699	483	6	33773	4.7
40	262.0	1385.0	<0.001	5.3	Naphthoate synthase	gi | 29375076	266	3	29847	5.2
41	423.8	2916.0	0.007	6.9	ATP-dependent protease peptidase subunit	gi | 29376201	240	2	19700	4.9
42	68.0	3805.6	0.019	55.9	Hypothetical protein EF1753	gi | 29376302	656	7	59558	4.7
43	357.9	3445.1	0.001	9.6	ABC transporter ATP-binding protein	gi | 29376561	423	4	27612	4.9
44	134.5	4480.4	0.002	33.3	Hypothetical protein EF1753	gi | 29376302	454	5	59558	4.7

47	498.6	1072.7	0.039	2.2	Chaperonin, 33 kDa	gi | 29374907	795	8	32261	4.7
48	640.4	1332.1	0.050	2.1	HAD superfamily hydrolase	gi | 29375718	400	4	30053	4.9
49	782.5	1966.5	0.022	2.5	Hypothetical protein EF1402	gi | 29375969	462	5	16970	6.0
50	562.8	1472.4	0.019	2.6	Threonine synthase	gi | 29376915	757	7	37139	5.5
51	442.4	1584.0	0.016	3.6	Metallo-beta-lactamase superfamily protein	gi | 29376926	278	2	23172	5.1
52	52.3	797.7	0.017	15.2	Cobyric acid synthase	gi | 29377073	224	2	25821	4.9
53	156.9	627.6	0.019	4.0	Cro/CI family transcriptional regulator	gi | 29377418	163	2	36988	5.1
Down-regulated proteins										
2	9248.1	1517.9	0.012	0.2	Hypothetical protein EF2923	gi | 29377387	343	3	8531	4.6
4	3521.5	352.4	0.076	0.1	Fumarate reductase flavoprotein subunit	gi | 29377044	833	7	53781	5.3
5	12048.4	3091.1	0.018	0.3	30S ribosomal protein S6	gi | 29374668	196	2	11598	5.0
6	21379.7	7394.6	0.028	0.3	Phosphocarrier protein HPr	gi | 29375306	94	1	9315	4.9
7	21695.5	6762.8	0.053	0.3	50S ribosomal protein L31	gi | 29375747	227	2	10119	5.6
8	8903.0	1581.2	0.082	0.2	50S ribosomal protein L7/L12	gi | 29377194	787	5	12389	4.5
9	1547.1	600.7	0.090	0.4	Phosphopentomutase	gi | 29374833	403	4	42773	4.9
10	1134.6	589.4	0.033	0.5	Ribulose-phosphate 3-epimerase	gi | 29377574	440	3	23661	4.7
12	3004.3	1680.5	0.058	0.6	Amino acid ABC transporter ATP-binding protein	gi | 29375353	192	2	26906	4.8
13	1376.6	795.8	0.090	0.6	Pheromone cAD1 lipoprotein	gi | 29377699	262	3	33122	5.3
14	2588.7	1610.9	0.034	0.6	3-oxoacyl-ACP synthase	gi | 29377346	839	7	43043	5.1

[a] spot numbers as given in supplementary Figure S1

[b] represents the average spot quantity for three control biological replicates .

[c] represents the average spot quantity for three bacitracin-stressed biological replicates.

[d] represents the probability associated with Student's t-test (two tailed and paired) tested between the 3 control samples and 3 bacitracin stressed samples.

[e] represents the x-fold change in spot quantity between the bacitracin stressed and the control samples.

[f] Proteins with one peptide match were manually confirmed by the Centre for Protein Research, University of Otago.

Note that spots number 25, 32, 34, 39 and 46 with increased and spots number 1, 3, 11 and 45 with decreased quantity had no significant protein hits in MASCOT (Matrix Science) analysis and thus are not included in this table.

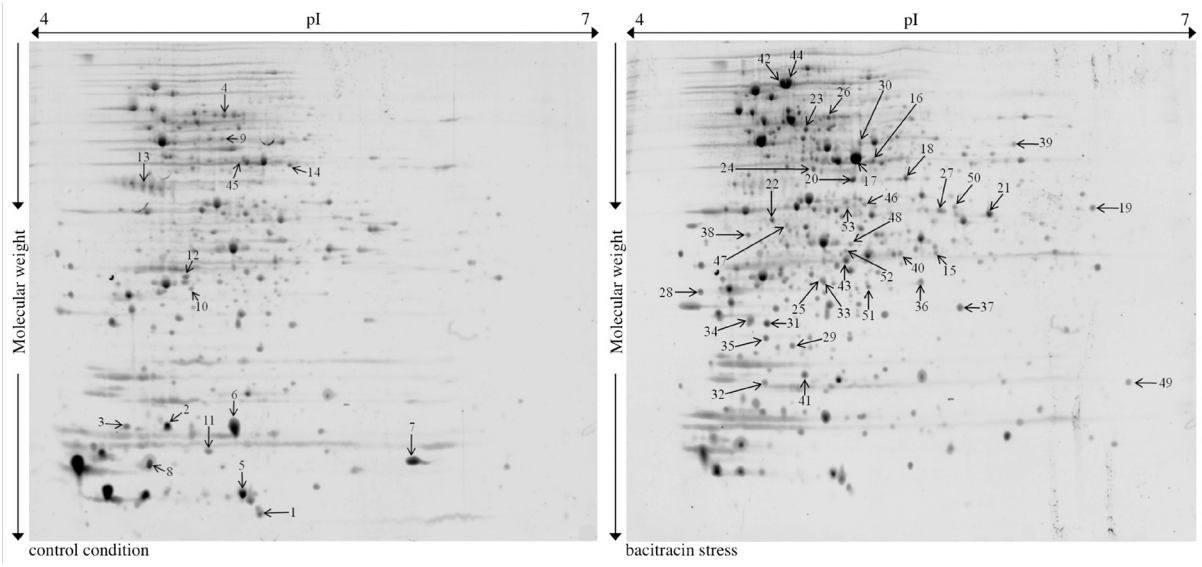

FIG S1 Proteomic analysis of the bacitracin stress response of *E. faecalis* V583. Protein extracts of untreated cells (left gel) or cells treated with 64 µg ml^{-1} bacitracin (right gel) were separated in the first dimension by isoelectric focusing on an 18 cm pH 4-7 ReadyStrip™ IPG strip (Bio-Rad) and in the second dimension by SDS-PAGE (12.5 % acrylamide). Proteins were stained with SYPRO-Ruby (Invitrogen) and the gel image was acquired using a Molecular Imager FX (Bio-Rad). On the left gel, protein spots with decreased intensity after bacitracin exposure are labelled, on the right gel, protein spots with increased intensity. Detailed information on spot identification is given in supplementary Table S4 and a summary in Table S3, using the same spot numbering as shown in this figure.

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