HHS Public Access

Author manuscript

Chem Phys Lipids. Author manuscript; available in PMC 2020 August 01.

Published in final edited form as:

Chem Phys Lipids. 2019 August ; 222: 23–35. doi:10.1016/j.chemphyslip.2019.04.010.

A novel synthesis of *trans*-unsaturated fatty acids by the Gram-positive commensal bacterium *Enterococcus faecalis* FA2–2

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Abstract

A key mechanism of *Pseudomonas* spp. adaptation to environmental stressors is their ability to convert the *cis*-unsaturated fatty acids of the membrane lipids to their *trans*-isomers to rigidify the membrane and thereby resist stresses. Although this Cti-catalyzed enzymatic isomerization has been well investigated in the *P. putida* paradigm, several bacterial species have been found to produce *trans*-unsaturated fatty acids. Although *cti* orthologs have only been reported in Gram-negative bacteria, we report that *E. faecalis* FA2–2 cultures synthesize *trans*-unsaturated fatty acids during growth by a mechanism similar of *P. putida*. Although the role of *trans*-unsaturated fatty acids *(trans*-UFAs) in *E. faecalis* remains obscure, our results indicate that organic solvents, as well as the membrane altering antibiotic, daptomycin, had no effect on *trans*-UFA formation in *E. faecalis* FA2–2. Moreover *trans*-UFA production in *E. faecalis* FA2–2 membranes was constant in oxidative stress conditions or when metal chelator EDTA was added, raising the question about the role of heme domain in *cis-trans* isomerization in *E. faecalis* FA2–2. Although growth temperature and growth phase had significant effects on *cis-trans* isomerization, the bulk physical properties of the membranes seems unlikely to be altered by the low levels of *trans*-UFA. Hence, any effects seems likely to be on membrane proteins and membrane enzyme activities.

We also report investigations of *cti* gene distribution in bacteria was and suggest the distribution to be triggered by habitat population associations. Three major Cti clusters were defined, corresponding to *Pseudomonas, Pseudoalteromonas* and *Vibrio* Cti proteins.

Keywords

cis-trans isomerase; *E. faecalis*; fatty acid; membrane; adaptation; heme

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Conflict of Interest

We have no conflicts of interest to declare.

1. Introduction

In response to stress many bacteria have evolved different mechanisms in order to adapt and survive the changing environment. The cell membrane of bacteria is typically the first barrier between the environment and the bacterial cell. Several stressors, such as temperature increase or addition of organic solvents directly increase membrane fluidity, leading to the disruption of essential membrane functions (Hermann J. Heipieper et al., 2003; Heipieper and de Bont, 1994). As a result, bacteria have developed mechanisms to alter cell membrane fluidity in order to maintain constant fluidity in the presence of environmental stress (Sinensky, 1974). One such mechanism is the post-synthetic transformation of membrane *cis*-unsaturated fatty acids (*cis*-UFA) into their *trans* isomers (*trans*-UFA) (von Wallbrunn et al., 2003). The benefit of this conversion is based on steric differences between *cis*- and *trans*-UFA. The double bonds of a *cis*-UFA form a kinked steric structure resulted in highly fluid membranes. In contrast, the more extended *trans*-UFA orders the membrane decreasing fluidity relative to their *cis*-isomers (McDonough et al., 1983; Seelig and Waespe-Sarcevic, 1978). Enzymatic isomerization of *cis*-UFA to *trans*-UFA is catalyzed by Cti and does not depend on *de novo* FA and protein synthesis, ATP or any other cofactor (Heipieper et al., 2003; Pedrotta and Witholt, 1999), presenting an efficient means to rigidify the membrane in the response to changing environments. Cti is a periplasmic cytochrome *c*-type protein (Pedrotta and Witholt, 1999) and containing a covalently bound heme essential for the *cis* to *trans* isomerization reaction (Holtwick et al., 1999). The iron provided by heme domain was proposed to remove the electrons from the *cis* double bond and then reconstitute the double bond in lower energy *trans* configuration without its transient saturation (Heipieper et al., 2003; von Wallbrunn et al., 2003).

The production of *trans*-UFA has been well described in various *Pseudomonas putida* strains where *trans*-UFA are reported to play an important role in adaptation of diverse *P. putida* strains to temperature increase, presence of organic solvents and heavy metals, as well as osmotic stress and addition of membrane-active antibiotics (Heipieper et al., 1996; Isken et al., 1997; Neumann et al., 2003). However, as summarized in a recent review, *cis-trans*-isomerization was shown in strains of *Pseudomonas* sp., *Vibrio* sp., *Methylococcus capsulatus*, *Alcanivorax borkumensis*, and *Colwellia psychrerythraea*. However, the presence of Cti orthologs have been found in other microorganisms (Eberlein et al., 2018; Heipieper et al., 2010).

Enterococcus faecalis is a Firmicute (Gram-positive) bacterium, found in the gastrointestinal tract of mammals and is one of the leading causes of surgical wound infections (Huycke et al., 1998). Unlike Gram-negative bacteria, Gram-positive bacteria lack an outer layer (wall) and periplasm (Silhavy et al., 2010) and have not been reported to produce *trans*-UFA. Here, we have investigated the *cti* gene distribution among bacterial species and found that only 5.5% of tested bacterial strains code for *cti* orthologs and all were Gram-negative bacteria found in environments membrane altering stressors are present. However, our results showed that *E. faecalis* FA2–2 forms *trans*-UFA during growth using the pattern similar to seen in *P. putida*. Nonetheless, the oxidative stress and the metal chelation had no effect on *E. faecalis* FA2–2 *trans*-UFA production. Whereas the role of these FA in *E. faecalis* membrane is

unclear, organic solvents, as well as daptomycin, had no effect on *trans*-UFA formation in *E. faecalis* membranes.

2. Material and Methods

2.1 Strains and growth conditions

Bacterial strains used in this study are listed in Table S1. *P. putida* F1 and *E. faecalis* FA2–2 were grown at 30°C and 37°C, respectively, in M17 (BD Difco) fatty acid free broth, obtained by three chloroform (v/v) extractions of FA from the medium. When indicated, M17-FA free was supplemented with *9,10*-D_2-oleic acid or 0.09% sodium [1-^{13}C]acetate both from Cambridge Isotope Laboratories. For *P. putida* F1, 9,10-D_2-oleic acid was neutralized with KOH, solubilized with Tergitol NP-40 and added at final concentrations of 0.01%. For *E. faecalis* FA2–2, *9,10*-D_2-oleic acid was added at a final concentration of 100 μM. When treated with daptomycin, bacterial cultures were supplemented with 50 mg of Ca^{2+} /liter. Minimum inhibitory concentrations (MICs) were determined by the broth microdilution method achieved in M17 FA free. Briefly, overnight cultures were diluted to OD_{600} of 0.08 and added to a 96-well test plate (Nunc) containing different concentrations of stressors in triplicate. The test plates were incubated at 30°C or 37°C for overnight. MIC was defined as the lowest antibiotic concentration that inhibited bacteria growth as determined by turbidimetry at OD_{600}.

2.2 Fatty acid analyses

Phospholipids were extracted (Bligh and Dyer, 1959) and fatty acid methyl esters (FAME) were prepared according to a standard protocol (Zhu et al., 2010). Briefly, the phospholipids were dissolved in 1.2 mL of dry methanol. Esterification reaction was conducted by incubation with 0.2 mL of 25% (v/v) sodium methoxide at room temperature for 15 min and stopped by addition of 1.2 mL of 2 M HCl. FAME were then obtained by three extractions each with 1.2 mL of hexanes. The solvent was removed under a nitrogen stream. Fatty acid methyl esters were analyzed using a GC-MS system (Agilent Inc, CA, USA) consisting of an Agilent 7890B gas chromatograph, an Agilent 5977A MSD.

The *cis*- and *trans*-isomers were separated on a CP-Sil88 (50 m×0.25 mm I.D. and 0.2 μm film thickness) capillary column (Agilent J&W, CA, USA). The inlet temperature was 220°C, MSD interface temperature – 230°C, and the ion source temperature adjusted to 230°C. An aliquot of 1 μL was injected in a split mode (20:1). The helium carrier gas was kept at a constant flow rate of 1.9 mL min-1. The temperature program was: 2 min isothermal heating at 80°C followed by temperature increase of 10°C min^{-1} to 165°C, then 20°C min^{-1} to 180°C, 10°C min^{-1} to 210°C. The mass spectrometer was operated in positive electron impact mode (EI) at 69.9 eV ionization energy at m/z 33–500 scan range. Mass spectra were recorded in combined scan/SIM mode. For a SIM mode, following m/z fragments were tracked: 242 (C14:0), 236 (C16:1*cis,trans*), 250 (C17:0 cyclo), 264 (C18:1*cis,trans*), 270 (C16:0), 278 (C19:0 cyclo), 298 (C18:0). Obtained retention time was confirmed by authentic standards (Sigma, USA). Target peaks were evaluated by the Mass Hunter Quantitative Analysis B.08.00 (Agilent Inc., CA, USA) software. The results are presented as a % of total FA. All experiments were performed in three biological replicates.

2.3 Double bond localization

To locate double bonds dimethyldisulfide adducts the protocol previously reported (Feng and Cronan, 2009) was used. Fatty acid methyl esters in hexane (100 μl) were converted to their dimethyldisulfide adducts by treatment with 75 μl of dimethyldisulfide and one drop of 6%, iodine solution in diethyl ether for 14 h at 50°C. Samples were cooled, and 50 μl of 10% aqueous $Na2S_2O_3$ were added to remove iodine. The hexane layer was pooled and concentrated to 50 μl under N_2. Gas chromatography-mass spectroscopy analyses were done on an Agilent system consisting of a 5975C mass selective detector, a 7683B autosampler, and a 7890A gas chromatograph equipped with ZB-5MS (60 m×0.32 mm I.D. and 0.25μm film thickness) capillary column (Phenomenex, CA, USA). Injection temperature and the mass selective detector transfer line were set to 250 °C, the ion source and MS quadrupole were adjusted to 230 and 150°C, respectively. The helium carrier gas was set at a constant flow rate of 2 ml/min. The temperature program was: 2 min at 100°C, followed by an oven temperature increase of 8°C/min until 300°C. A 1 μl sample was injected with a split ratio of 10:1. The spectra acquired were recorded in the m/z 50–500 scanning range and processed using the Mass Hunter Quantitative Analysis B.08.00 (Agilent Inc., CA, USA) software.

2.4. Detection of *trans*-UFA in different *E. faecalis* FA2–2 lipid species

Lipids were extracted from *E. faecalis* FA2–2 pellets as described above and separated on HPTLC plates (silica gel 60 F_{254}, Sigma) using $CHCl_3/CH_3OH/CH_3COOH$ (65/25/10, v/v/v) as the solvent system. Individual lipid spots were visualized by UV fluorescence at 365 nm after spraying a primulin dye solution (0.05% in acetone/H_2O, 8/2, v/v). The identification of individual HPTLC spots was made in comparison to control HPTLC plates of known lipid standards. High purity lipid standards were obtained from Avanti Polar Lipids (Alabaster, AL).

To test the *trans*-UFA content in individual lipid types, the major lipid spots separated on the HPTLC plate were scrapped off and extracted using the Bligh and Dyer method. Next, FAME were prepared and analyzed using GC MS as described above.

2.5 Phylogenic analyses

The closest Cti orthologs were extracted from the MaGe Platform (http://www.genoscope.cns.fr/agc/mage, last accessed June 10, 2018), which carried out Blast analyses using the *P. putida* F1 Cti amino acid sequence (*Pput_3319*) as a query in BLAST-P searches. We thoroughly checked all available genomes of species lacking *cti* to ensure absence of the gene itself or lack of any significant remnant. Whenever possible, we also favored fully assembled genomes over draft or incomplete ones. The resulting sequences were analyzed using the neighbor-joining method using CLC Sequence Viewer 7 (CLC bio). Bootstrap values at nodes greater than 80% (1000 replicates) were chosen to construct the tree.

3. Results

3.1 *E. faecalis* FA2–2 forms *trans*-UFA during growth

Despite the absence of Cti orthologous genes in Gram-positive bacteria, we tested if a Gram-positive bacterium might produce *trans*-UFA. We analyzed the FA composition of early stationary growth cultures of *E. faecalis* FA2–2 grown in M17 FA free broth and found 1.6% of the total bacterial FA was *trans*-UFA (Table 1). In cultures of *P. putida* F1 grown under the same conditions, 20.2% *trans*-UFA from total FA was found. However, in cultures of *E. coli* MG1655, a bacterium known to be unable to produce *trans*-UFA, as well as a *cti* mutant of *P. putida* F1 (Kondakova and Cronan, 2019) *trans*-UFA were not detected.

To ask if the *trans*-UFA found in *E. faecalis* FA2–2 cultures were synthesized by the bacterium rather than taken up from the medium, sodium [1-^{13}C]acetate was added to bacterial cultures. Incorporation of ^{13}C into C16:1-*trans* UFA would indicate that the fatty acid was synthesized from (ultimately) acetyl-CoA rather than derived from the medium. Indeed, ^{13}C labeled C16:1-*trans* UFA were detected in *E. faecalis* FA2–2 and *P. putida* F1 cultures, but not in *P. putida* F1 *Δcti* cultures (Fig. 1A). The MS spectra of C16:1-*trans* showed the enrichment in ^{13}C compared to the spectrum of C16:1-*trans* standard (Fig. 1B), demonstrating the production of *trans*-UFA by *E. faecalis* FA2–2.

We next tested if the production of *trans*-UFA occurred during *E. faecalis* FA2–2 growth or during the sample preparation, as was previously reported for several *P. putida* strains (Härtig et al., 2005). Bacterial cultures were treated with the powerful chaotrophic agent 10% trichloroacetic acid (TCA) as previously described (Kondakova and Cronan, 2019) (Fig. S1A). Fatty acid methyl esters (FAME) were extracted from the TCA precipitates and separated using a CP-Sil 88 column which gave a well-separated peak corresponding to C16:1-*trans* in both the *P. putida* F1 and *E. faecalis* FA2–2 samples (Fig. S1B). No *trans*-UFA was detected in *E. coli* K-12 MG1655 samples given the same treatment, indicating that the formation of C16:1-*trans* is not an artefact of TCA treatment. Altogether, these data indicated that *E. faecalis* FA2–2 synthesized *trans*-UFA during growth rather than during the stress of handling.

3.2 *E. faecalis* FA2–2 produces C16:1-*trans* Δ*9* using *cis*-UFA as a substrate utilizing a mechanism similar to that of *P. putida* spp.

To determine the location of the *E. faecalis* C16:1-*trans* fatty acid double bond we performed derivatization of fatty acid methyl esters (FAMEs) with dimethyldisulfide as previously reported (Feng and Cronan, 2009). Both the C16:1-*cis* and C16:1-*trans* UFAs gave adduct peaks corresponding to the Δ*9* position of the double bond (Fig. 2). These data confirmed the C16:1-*trans* identification in *E. faecalis* FA2–2 samples and showed that *trans*- and *cis*-double bonds are located in the same position of the acyl chain.

Previous reports investigated the pattern of the *cis*- to *trans*-UFA isomerization in *P. putida* S12 showing that Cti-mediated isomerization did not include the transient saturation of the double bond (von Wallbrunn et al., 2003). To test if in *E. faecalis* FA2–2 *cis*-UFA are the substrate for *trans*-UFA formation and if this isomerization reaction occurs without double bond saturation, bacterial cultures were supplemented with oleic acid (C18:1-*cis* Δ*9*)

deuterated at both C atoms of the double bond (D_2-C18:1-*cis* Δ*9*). In agreement with previous study (von Wallbrunn et al., 2003), *P. putida* F1 samples showed four C18:1 FA (Fig. 3A top) species; two native species, C18:1-*cis* Δ*11* and C18:1-*trans* Δ*11* (Fig. 3B peaks 4 and 2, respectively, m/z 296.3 indicating no deuteration), which are known to be formed using the anaerobic pathway for UFA synthesis (Cronan, 2006; Cronan and Thomas, 2009) and two nonnative doubly deuterated UFAs, D_2-C18:1-*cis* Δ*9* and D_2-C18:1-*trans* Δ*9* FA (Fig. 3B peaks 3 and 1, respectively, m/z 298.3, indicating double deuteration). No *trans*-UFA were detected in *P. putida* F1 *Δcti* cultures, showing the absence of sample contamination during the experiments. *E. faecalis* FA2–2 *ΔfabI* cultures fed with deuterated oleic acid did not produce any detectable amount of native C18:1-*cis* Δ*11* and C18:1-*trans* Δ*11* FA, due to the blockage of the FA biosynthetic pathway by the *ΔfabI* mutation (Zhu et al., 2013), but readily incorporated the supplemented D_2-C18:1-*cis* Δ*9* and converted it to the *trans*-isomer, D_2-C18:1-*trans* Δ*9* FA, (Fig. 3A bottom panel), indicating that *E. faecalis* FA2–2 directly converted *cis*-UFA to *trans*-UFA. The mass fragmentation pattern of D_2-C18:1-*trans* Δ*9* in these samples showed a C18:1-*trans* Δ*9* product completely labeled with two deuterium atoms demonstrating that no deuterium was lost during the isomerization. These data demonstrate that *E. faecalis* FA2–2 produced *trans*-UFA using *cis*-UFA as a substrate without shift of double bond position or transient saturation of the double bond.

3.3 *trans*-UFA are detected in all *E. faecalis* FA2–2 lipids, except lyso-phosphatidylglycerol.

To ask in which lipid species *trans*-UFA are located, we first studied the *E. faecalis* FA2–2 lipid composition. Enterococcal lipid compositions have been previously described in detail (Bao et al., 2012; Mishra et al., 2012; Rashid et al., 2017) and the predominant *E. faecalis* lipid species are phosphatidylglycerol (PG) and cardiolipin (CL). However, small amounts of phosphatidylethanolamine (PE) and phosphatidic acid were also reported. This bacterium is also able to modify PG with lysine to produce lysyl-PG and thereby modify the membrane charge (Bao et al., 2012). In addition, *E. faecalis* has been shown to contain several glycolipids including phosphatidyldiglycosyldiglyceride and glycerophosphoryl-diglycosyl-diglyceride (Mishra et al., 2012), as well as diglycosyl-diacylglycerol (DGDAG), dyacylglycerols (DAG) and triacylglycerols (TAG) (Rashid et al., 2017). To identify the major *E. faecalis* FA2–2 lipid species, the total FA2–2 lipids were separated on silica gel HPTLC plates and identified by comparing with HPTLC plates of lipid standards (Fig 4A). Two major lipid spots were identified as PG (Rf of 0.76) and DGDG coeluted with PE (Rf of 0.60), (Fig. 4C). However, three smaller spots were also detected and identified as lysyl-PG (Rf of 0.07), lyso-PG (Rf of 0.28) and CL probably co-eluted with DAG (Rf of 0.91).

To ask which lipid types contain *trans*-UFA, the appropriate areas of silica gel were scrapped from the HPTLC plates, extracted and hydrolyzed to produce FA. The FA were then methylated and analyzed by GC MS. This method was first tested for extraction of *cis*- and *trans*-UFA from PG standard separated or not on HPTLC plate. About 90% and 81% of *trans*-UFA and *cis*-UFA (non-HPTLC treated control was 100%) were extracted from HPTC plate and detected by GC MS (Fig. 4B). Next, assayed for *trans*-UFA in all *E. faecalis* FA2–2 lipids. Interestingly, *trans*-UFA were detected in all tested spots, except that of lyso-PG, indicating that *E. faecalis* FA2–2 lyso-PG does not contain *trans*-UFA. PG is the major PL

and as expected this PL contained most of the *trans*-UFA (Fig. 4D). Together these data indicated that *trans*-UFA is present in all the tested major *E. faealis* FA2–2 lipids, excepting lyso-PG.

3.4 Effect of growth phase and temperature on *trans*-UFA production in *E. faecalis* FA2–2

We evaluated the effects of growth phase and temperature on *E. faecalis* FA2–2 *trans*-UFA production. The fraction of *trans*-UFA in *E. faecalis* FA2–2 membrane significantly increased from exponential to early stationary growth phase, where the maximum *trans*-UFA levels were seen (Fig. 4A&B). The fraction of *trans*-UFA in *E. faecalis* FA2–2 membranes then modestly decreased to become stable after 36 h of growth. The same trend has been observed in *P. putida* F1 cultures (Kondakova and Cronan, 2019), as well as in *Vibrio* sp. strain no. 5710 cultures, which showed maximal of *trans*-UFA levels in early stationary growth phase (Hamamoto et al., 1994). This indicated a possible correlation between the production of *trans*-UFA and growth phase in these bacterial species.

Previous studies showed that the production of *trans*-UFA plays an important role in *P. putida and Vibio* spp. adaptation to temperature increase (Diefenbach et al., 1992; Holtwick et al., 1997; Okuyama et al., 1991, 1990) and is considered as a fast-adaptive response of bacterial membranes to temperature change. Thus, we tested the temperature shift effect on production of *trans*-UFA in *E. faecalis* FA2–2. As reported by (Diefenbach et al., 1992) and our previous study (Kondakova and Cronan, 2019), bacterial cultures were grown at optimal growth temperature 37°C until the early stationary growth phase (point #3 Fig. 4A) and then the growth temperature was shifted to 20°C, 30°C or 42°C for 2 hr. Whereas the fraction of *trans*-UFA did not change when temperature was shifted at 20°C and 30°C, the amount of *trans*-UFA in *E. faecalis* FA2–2 cultures grown at 30°C was significantly higher that seen in cultures grown at 42°C (Fig. 4C).

3.5 The levels of *trans*-UFA in *E. faecalis* FA2–2 is independent of *de novo* protein synthesis

It was previously proposed that *trans*-UFA synthesis in *P. putida* does not depend on the *de novo* protein synthesis (Heipieper et al., 1992). To ask if the production of *trans*-UFAs in *E. faecalis* FA2–2 depends on *de novo* protein biosynthesis, we exposed *E. faecalis* FA2–2 and *P. putida* F1 cultures to chloramphenicol (Aakra et al., 2010) for 2 h in concentrations above the minimal inhibitory concentration (10 μg/mL for *E. faecalis* FA2–2 and 550 μg/mL for *P. putida* F1). In agreement with previous study (Heipieper et al., 1992), there was no significant change in *trans*-UFA production in either strain (Fig. 5H), indicating that production of *trans*-UFAs in *E. faecalis* FA2–2 does not depend on *de novo* protein biosynthesis. It should be noted that after 2 h exposure both bacterial strains remained viable (Fig. S2G).

3.6 Oxidative stress and addition of the EDTA chelating agent does not modify *trans*-UFA production in the *E. faecalis* FA2–2 membrane

In *P. putida* Cti is a cytochrome *c*-type protein that has the characteristic covalently bound heme-binding motif (Holtwick et al., 1997), the iron of which (probably Fe^{3+}) was proposed to be essential for the *cis*- to *trans*-isomerization (Okuyama et al., 1998). When added to

bacterial cultures, paraquat is known to produce oxidative stress by generating reactive oxygen species (Lascano et al., 2012; Mancini and Imlay, 2015), which reduce cytochrome *c* (Koppenol et al., 1976; Lascano et al., 2012) and could inhibit Cti-dependent production of *trans*-UFA. Thus, we tested 2 h treatments of both *P. putida* F1 and *E. faecalis* FA2–2 cultures with increasing concentrations of paraquat. *E. faecalis* FA2–2 showed no significant decrease in *trans*-UFA production as compared to *P. putida* F1 which showed a significant decrease in *trans*-UFA production (Fig. 5D). Paraquat is a cation which cannot passively diffuse across the membrane and needs to be taken into the cell via transporters (Lascano et al., 2012). To insure the paraquat penetration into *E. faecalis* FA2–2 cells, we analyzed the time course of 500 μM paraquat effect on *trans*-UFA production in this bacterium. Even after 5 h of incubation time there was no significant change in *trans*-UFA production (Fig. 5E). This indicates that in contrast to *P. putida* F1, in *E. faecalis* FA2–2 reactive oxygen species did not affect *trans*-UFA production.

Ethylenediaminetetraacetic acid (EDTA) is one of the most effective chelating agents and forms an open complex with Fe^{3+} ion (Flora and Pachauri, 2010; Maketon et al., 2008) and thus EDTA could compete with a heme binding site for the metal. To ask if the EDTA-mediated metal chelation alters the production of *trans*-UFA in *E. faecalis* FA2–2, we exposed *E. faecalis* FA2–2 and *P. putida* F1 cultures to increasing concentrations of EDTA for 2 h. There was a significant decrease in *trans*-UFA production in *P. putida* F1, however there was no decrease in *trans*-UFA production in *E. faecalis* FA2–2 (Fig. 5F), indicating that chelating agent such as EDTA does not affect *trans*-UFA production in *E. faecalis* FA2–2.

Together these data showed that in contrast to *P. putida* F1, *trans*-UFA production in *E. faecalis* FA2–2 did not decrease in oxidative stress conditions or in presence of the chelating agent, EDTA, suggesting that the *E. faecalis* isomerization enzyme(s) did not have *P. putida* Cti characteristics.

3.7 The levels of *trans*-UFA in *E. faecalis* FA2–2 membrane unaffected by exposure to organic solvents or daptomycin

Next, we investigated the role of *trans*-UFA production in *E. faecalis* FA2–2. The importance of membrane *trans*-UFA in maintaining constant membrane fluidity was well documented for several *P. putida* strains, which overproduce *trans*-UFAs to decrease membrane fluidity in the response of organic solvents, such as toluene or octanol (Heipieper et al., 1996, 1995; Heipieper and de Bont, 1994; Junker and Ramos, 1999; Pedrotta and Witholt, 1999). To test whether *E. faecalis* FA2–2 *trans*-UFA production would increase when exposed to organic solvents, we subjected it to octanol and toluene treatment. Hence, cultures in early stationary growth phase were incubated for 1 h with increasing concentrations of octanol and toluene (Fig. 5A). No significant increase in *trans*-UFA production was observed when bacterial cultures were incubated with toluene or octanol (Fig. 5B&C) indicating that exposure to these organic solvents did not affect *E. faecalis* FA2–2 *trans*-UFA production. Surprisingly, *E. faecalis* FA2–2 was found to tolerate high concentrations of octanol and toluene. In fact, it grew even after 1 h exposure to 4.5 mM and 35 mM octanol and toluene, respectively (Fig. S2A&B).

Daptomycin (DAP) is a lipopeptide antibiotic effective against Gram-positive bacteria such as *E. faecalis*, which interacts with the cell membrane lipids (Jung et al., 2004; Straus and Hancock, 2006). In complex with Ca^{2+}, DAP has an increasing affinity for the negatively charged phospholipids, including phosphatidylglycerol, which is a major component of the *E. faecalis* membrane lipids (Mishra et al., 2012; Rashid et al., 2017). In addition, DAP binds and clusters fluid lipids, such as *cis*-UFA (Müller et al., 2016). To test if DAP treatment of *E. faecalis* FA2–2 would change the level of *trans*-UFA in the membrane, we treated *E. faecalis* FA2–2 for 1 h with increasing DAP concentrations and measured the *trans*-UFA content in both the bacterial pellet and supernatant. The supernatant was analyzed because *E. faecalis* is known to release phospholipids to inactivate DAP (Mishra et al., 2012). DAP concentrations below and above the MIC (60 μg/mL) were tested. Neither the supernatant nor the pellet treated with DAP showed any change in *trans*-UFA production compared to the untreated cells (Fig. 5G), indicating that DAP has no effect on *trans*-UFA production in *E. faecalis* FA2–2.

Together these results indicated that the production of *trans*-UFA in *E. faecalis* FA2–2 was independent of the addition of organic solvents or membrane altering agents, such as daptomycin, suggesting that *trans*-UFA are not involved in the protection of bacterial membranes against the tested stress factors and likely play another role in *E. faecalis*.

3.8 Cti distribution among bacterial kingdoms

As summarized in a recent review (Eberlein et al., 2018), the presence of *cti* orthologous genes have been found in various bacterial strains. To investigate Cti distribution among bacterial species, we performed BLASTP analysis on translated reading frames of 3912 bacterial genomes available in MaGe database using the *P. putida* F1 Cti sequence (Pput_3319) as the query. Only 217 candidates were found to code for *P. putida* F1 *cti* orthologs (Table S2). All Cti encoding bacteria are Gram-negative bacteria belonging to Alpha-, Beta-, Delta- or Gamma subdivisions and use either aerobic or anaerobic respiration (Table S3). The majority of *cti* coding bacteria were found in high osmolarity marine environments, as well as environments contaminated by hydrocarbons, heavy metals or other toxic membrane-altering compounds, indicating that the environment could be an important driving force for *cti* distribution among bacterial kingdom.

To map the phyletic patterns of Cti proteins, we reconstructed a phylogenic tree from a concatenated alignment of all found sequences (Fig. 6 and Table S2). Three major Cti clusters were found representing *Pseudomonas* spp. Cti; *Pseudoalteromonas* spp. Cti and *Vibrio* spp. Cti. Whereas *Pseudoalteromonas* and *Vibrio* clusters had about 50% Cti amino acid sequence identity between each other, *Pseudomonas* Cti has only 35–39% identity with *Pseudoalteromonas* and *Vibrio* Cti clusters. The *Pseudomonas* Cti cluster was homogenous, including all available in MaGe database *Pseudomonas* spp. Cti species, as well as Cti from *Azotobacter vinelandii*, which is phylogenetically closely related to *Pseudomonas* genus (Özen and Ussery, 2012). All available into the database *Pseudoalteromonas* species coded for *cti*, which were clustered in *Pseudoalteromonas* Cti cluster. Only Cti from *Pseudalteromonas atlantica* T6c and *Pseudoalteromonas* sp. PLSV appeared in the *Vibrio* Cti cluster. Whereas the *Vibrio* Cti cluster was manly composed of *Vibrio* spp. Cti, not all *Vibrio*

species available in the database were found to code for *cti*. For instance, Cti was not found in several *V. cholerae*, *V. fischeri*, *V. vulnificus* and *V. harveyi* strains, indicating the heterogenic distribution of Cti among *Vibrio* genus. Instead, the *Vibrio* Cti cluster contained Cti encoding genes of two *Photobacterium* spp., five *Alteromonas* spp., two *Shewanella* spp. and *Rheinheimera* sp. EpRS3. Although *Photobacterium* belongs to the order Vibrionales, the presence of *Alteromonas* and *Shewanella* Cti coding genes in the *Vibrio* cluster rather than the phylogenetically closer *Pseudoalteromonas* cluster (*Pseudoalteromonas* and *Shewanella* belong to the order Alteromonadales) indicates the possible occurrence of horizontal gene transfer. This is supported by the fact that only a few *Alteromonas* spp. (6 from 23 strains available into the database), *Photobacterium* spp. (2 from 10 strains) and *Shewanella* spp. (2 from 10 strains) were found to possess Cti coding sequences.

Several species that encode putative Cti proteins were not included in the three major clusters and formed individual branches or small groups of Cti orthologous proteins. Several of these Cti encoding genes were found in poorly studied bacterial genera, suggesting that with the steadily increasing number of sequenced bacterial genomes several additional Cti clusters could form. However, from the ten *Nitromonas* spp. available in the database, only two were found to code for *cti*, indicating that *Nitromonas* spp. probably obtained Cti through horizontal gene transfer and *Nitromonas* Cti proteins should probably be clustered with Cti species belonging to another genera. Interestingly, two strains, *Methylococcus capsulatus* Texas and *Cycloclasticus zancles* 7-ME, each had two *cti* gene copies. The *Methylococcus capsulatus* Texas two Cti sequences (#180 and #189) are similar to each other, whereas *Cycloclasticus zancles* 7-ME encoded Cti proteins having only 36% identity (#63 and #190, Fig. 6 and Table S1). Although Cti #63 was close to *Pseudomonas* Cti and had 45% identity with *P. putida* F1 Cti, Cti #190 neighbored the *Pseudoalteromonas* Cti cluster with 42% identity with Cti of *Pseudoalteromonas tunicate* D2 (#177).

Together, these data indicate that only 5.5% of sequenced bacterial genomes encode a Cti candidate and these form three major clusters.

4. Discussion

Our analyses of FA composition of Gram-positive commensal bacterium *E. faecalis* FA2–2 have shown the presence of *trans*-UFA in its membrane lipids, a first since *trans*-UFA had previously been reported only in Gram-negative bacteria. This bacterium synthetized C16:1-*trans*-UFA during growth using *cis*-UFA as a substrate. The *trans*- and *cis*-double bonds were located at $\Delta 9$ position, indicating that no shift in double bond position nor transient saturation of the double bond occurred during isomerization. These data agree with previous findings obtained with *P. putida* cultures (von Wallbrunn et al., 2003), indicating that *E. faecalis* probably uses an enzymatic mechanism similar to that of *P. putida* Cti to form *trans*-UFA, although no apparent Cti homologue is encoded in the *E. faecalis* genome. *Trans*-UFA were detected in all major *E. faecalis* FA2–2lipids. Only lyso-PG lacked *trans*-UFA. Together with the prior report that *trans*-UFA were located in the *sn*-2 position of the PL glycerol moiety in *Vibrio* ABE-1 (Okuyama et al., 1991) suggests that *E. faecalis* FA2–2 *cis/trans* isomerization occurs at the *sn*-2 position of PLs.

Since Cti in *P. putida* was reported have be a covalently attached cytochrome *c* type heme essential for the isomerization reaction (Holtwick et al., 1997; Okuyama et al., 1998), we tested the effect of oxidative stress caused by paraquat, as well as that of a chelating agent, EDTA, on the *trans*-UFA formation in *E. faecalis* FA2–2. Although, *P. putida* F1 cultures showed a decreased rate of *trans*-UFA into the membrane lipids when treated with either compound, *E. faecalis trans*-UFA rate was constant despite the treatment. One possible explanation could be heme inaccessibility in *E. faecalis cis-trans* isomerase.

Enterococci use an electron transport chain for aerobic respiration when heme is provided or, alternatively, can perform fermentation in the absence of heme. *E. faecalis* does not synthesize heme *de novo* and in contrast to pseudomonads has little or no requirement for nutritional iron (Cornelis and Dingemans, 2013; Keogh et al., 2017). However, in presence of heme *E. faecalis* cells were found to assemble two heme proteins, a membrane-bound cytochrome *bd* (Winstedt et al., 2000), and a cytoplasmic typical catalase (Baureder et al., 2014; Baureder and Hederstedt, 2012; Frankenberg et al., 2002). Thus, these proteins could be involved in *cis-trans*-isomerization in *E. faecalis*. We tested the FA composition of *E. faecalis* FA2–2 cultures, which grew in TSBG medium containing less than 0.05 μM heme and reported to unable *E. faecalis* V583 catalase activity (Frankenberg et al., 2002). These preliminary tests did not show any decrease of *trans*-UFA production in TSBG cultures comparing to heme supplemented cultures (data not shown). Although future investigations are needed to find the enzyme catalyzing *cis-trans* isomerization in *E. faecalis* FA2–2, we suggest these proteins are unlikely to be involved. This indicates that *trans*-UFA in *E. faecalis* FA2–2 could be formed by using a heme or other metal-dependent mechanism.

Although there are statistically significant differences in trans-UFA production between measurements at different temperatures and in different growth phases (Fig. 5), given the very modest trans-UFA content (ca. 2% of the total acyl chains) these differences are too small to affect bulk membrane physical properties. However, this does not preclude specific interactions with proteins or in signaling processes where the trans-UFA could play a physiological role(s).

In this study we also investigated possible physiological roles for *trans*-UFA production in *E. faecalis* FA2–2. In contrast to *P. putida, E. faecalis* FA2–2 did not increase *trans*-UFA production when organic solvents, such as octanol and toluene were added to bacterial cultures. This indicated that in this bacterium *trans*-UFA are not involved in membrane response to organic solvents. However, *E. faecalis* FA2–2 was found to exhibit surprising for Gram-positive bacteria solvent tolerance. Due to the inherent disadvantage of lacking an outer membrane, only a few Firmicute bacteria have been previously reported to exhibit solvent tolerance, including species of *Bacillus, Rhodococcus, Clostridium, Arthrobacter, Lactobacillus, Staphylococcus* and *Enterococcus* (Isken and de Bont, 1998; Na et al., 2005; Nielsen et al., 2005; Paje et al., 1997; Sardessai and Bhosle, 2002; Torres and Castro, 2003; Zahir et al., 2006). Some organic solvent tolerance mechanisms in Gram-positive bacteria have been proposed such as induction of general stress regulon; production of organic solvent emulsifying or deactivating enzymes; active solvent efflux pumps, as well as cell morphology alterations and filamentous growth (Torres et al., 2011). However *E. faecalis* is a ubiquitous commensal of mammalian gastrointestinal flora (Lebreton et al., 2014) and thus

unlikely to be naturally exposed to organic solvents. Thus, we tested the effect of daptomycin on the production of *trans*-UFA in *E. faecalis* FA2–2. Although daptomycin was reported to decrease membrane fluidity in *B. subtilis* and *E. faecalis* cells (Mishra et al., 2012; Müller et al., 2016), it had no effect on production of *trans*-UFA in *E. faecalis* FA2–2 cultures. It remains possible that *trans*-UFA production in *E. faecalis* could be in response to a specific stressor(s). that we have not tested, and the possibility that *E. faecalis trans*-UFA synthesis is not linked to membrane stress cannot be excluded.

Our analysis of Cti distribution among bacterial species showed that only about 5.5% of tested bacterial genomes contained *cti* coding genes. The detailed analysis of all bacterial species showed that the majority of them were found in environments containing membrane fluidity stressors suggesting that environment is an important factor in *cti* distribution. Among three Cti clusters reported in our study, the *Pseudomonas* and *Pseudoalteromonas* Cti clusters were homogenous whereas the *Vibrio* Cti cluster contained several Cti species from other bacteria species consistent with the known ability of these bacteria to take up DNA molecules This indicated that Cti could be evolutionary acquired via horizontal gene transfer, triggered by association in differential population habitats (Oliveira et al., 2017; Polz et al., 2013) which leads to a model of ecological Cti speciation. Nonetheless, in agreement with previous studies (Okuyama et al., 1998; Pedrotta and Witholt, 1999) we found *cti* orthologous genes only in Gram-negative bacteria.

5. Conclusions

In this study we investigated the distribution of *cti* coding sequence among bacterial species, showing that *cti* orthologs presented in only 5.5% tested bacterial strains. There are Gram-negative bacteria, the majority of which were found in contaminated or highly osmotic environments. However, we found that a Gram-positive bacterium *E. faecalis* FA2–2 formed *trans*-UFA using an undetermined pathway. The C16:1-*trans* Δ*9* were found to be formed during growth of the bacterium from C16:1-*cis* Δ*9*. The role of *trans*-UFA in *E. faecalis* membrane remains a puzzle, organic solvents, as well as daptomycin did not have significant effect on *trans*-UFA formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Alex Ulanov of the Metabolomics Center, University of Illinois for help with GC-MS analyses. This work was supported by National Institutes of Health Grant AI15650 from the National Institute of Allergy and Infectious Diseases.

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Highlights

- For the first time we demonstrate the production of *trans*-unsaturated fatty acids by a Gram-positive bacterium
- The mechanism of *trans*-UFA production was investigated.
- Although the role of *trans*-unsaturated fatty acids in *E. faecalis* FA2–2 remains unresolved, organic solvents, as well as daptomycin were unable to activate *trans*-UFA production in *E. faecalis* FA2-
- This work defines *cis-trans* isomerase (Cti) distribution among bacterial species. Three major Cti clusters were defined and a habitat associated *cti* distribution is proposed.

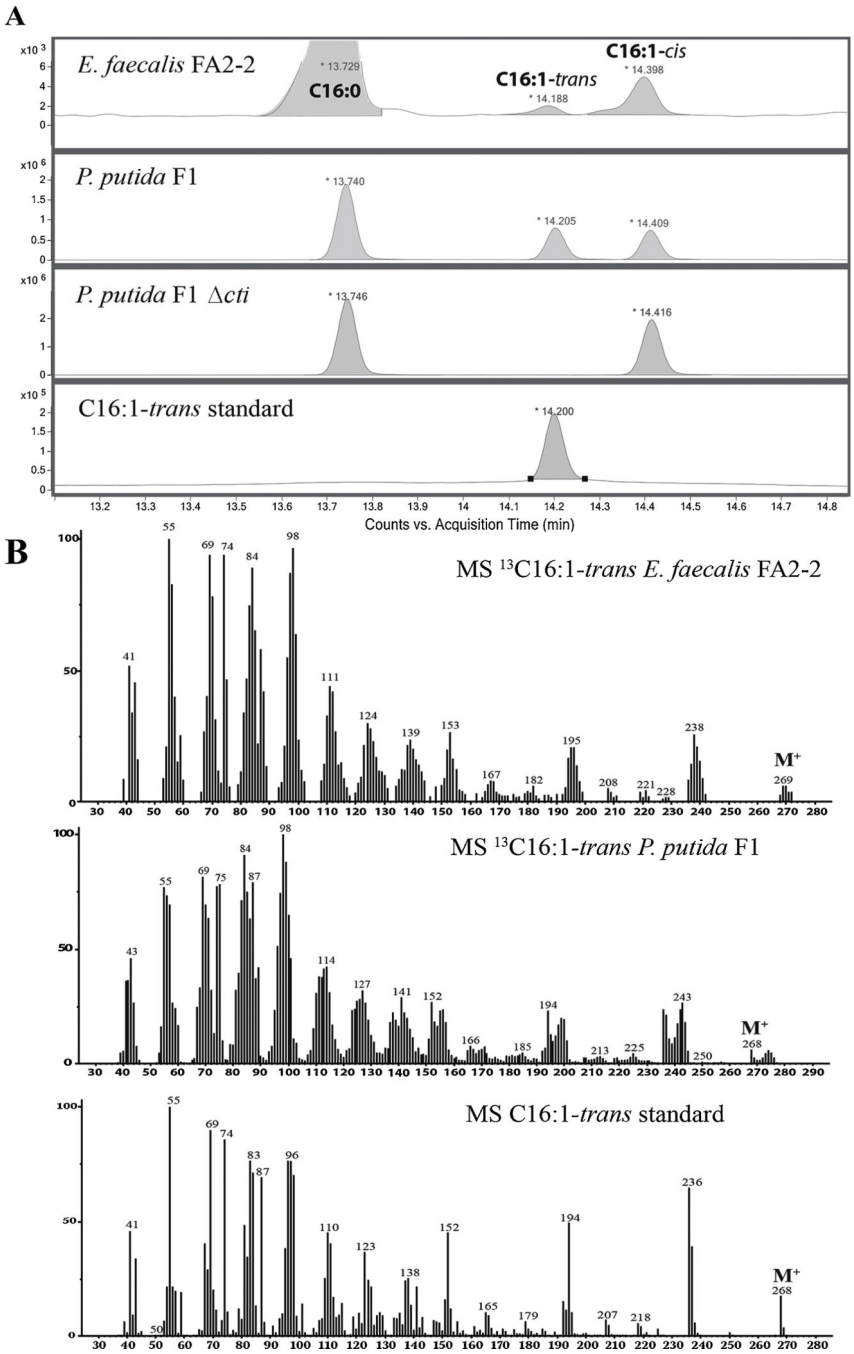

Figure 1. *E. faecalis* FA2–2 contains *trans*-UFA

(**A**) Representative total ion chromatograms of bacterial FAME showing that *E. faecalis* forms C16:1-*trans* fatty acids. Note the peak corresponding to C16:1-*trans* in *E. faecalis* FA2–2 and in *P putida* wild type, whereas this peak is absent in the *P. putida* F1 Δ*cti* strain. Bacteria were grown in M17-FA free medium supplemented with 0.09% sodium [1-^{13}C]acetate for 6.5 h and FAME were obtained as described in Material and Methods. N=3.

(**B**) Representative MS spectra showing the incorporation of sodium [1-^{13}C]acetate into C16:1-*trans* in *E. faecalis* FA2–2 (top) and *P. putida* F1 (middle). Note the large carbon isotopic distribution of C16:1-*trans* extracted from cultures of *E. faecalis* FA2–2 and *P. putida* F1 comparing to the C16:1-*trans* standard (bottom), which does not contain ^{13}C (N =3). For all panels, N indicates biological replicates corresponding to independent experiments.

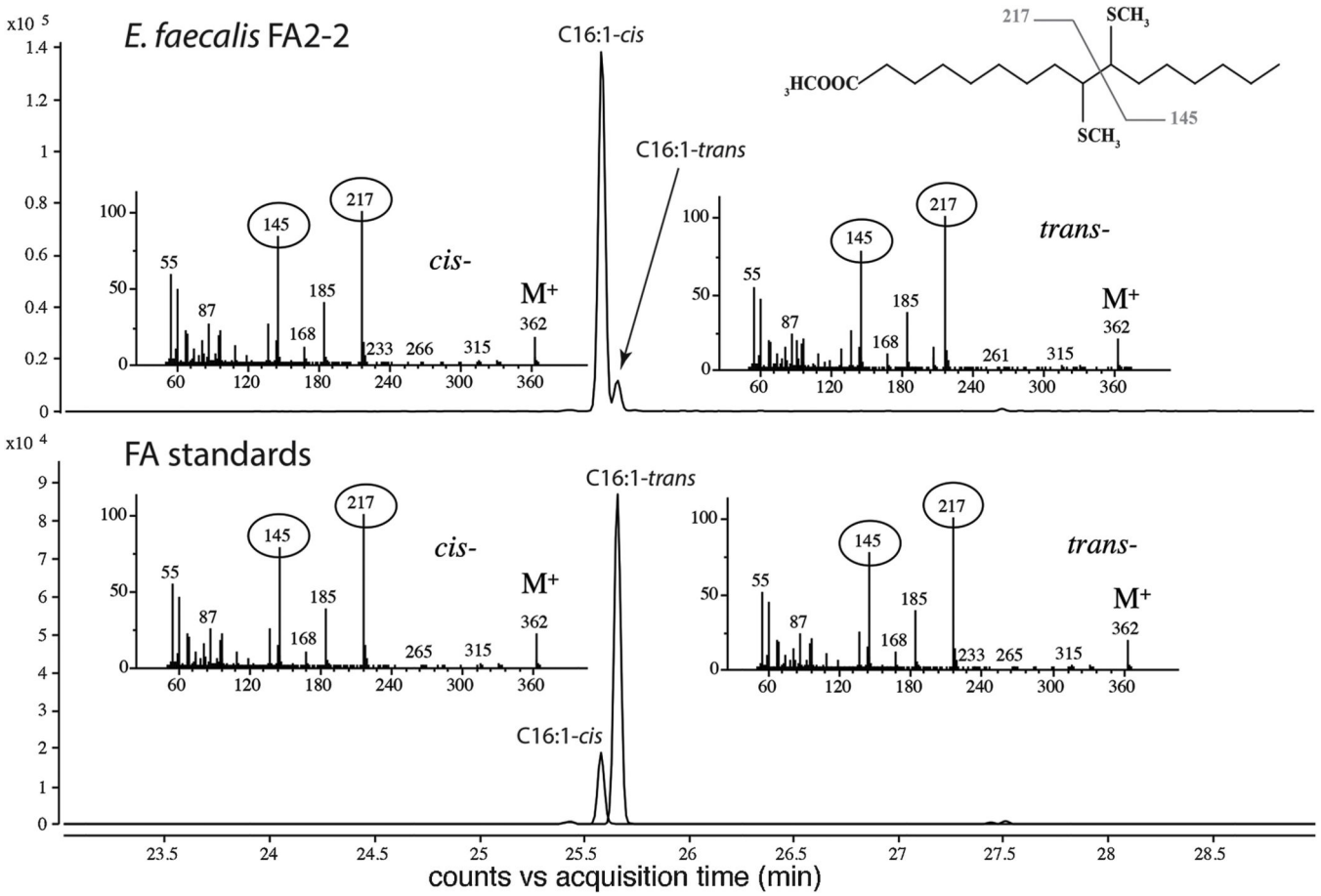

Figure 2. *E. faecalis* FA2–2 forms C16:1-*trans* Δ9

Representative total ion chromatograms of dimethyldisulfide adduct methyl esters showing that *E. faecalis* FA2–2 forms C16:1-*cis,transΔ9*. The localization of double bonds was assayed as reported in Material and Methods. The representative MS spectra of dimethyldisulfide adducts are shown by black arrows. The encircled fragments corresponding to methylsulfides were used to define the double bond position. Bacterial cultures in early stationary growth phase in M17-FA free medium were analyzed. N=3. N indicates biological replicates corresponding to independent experiments.

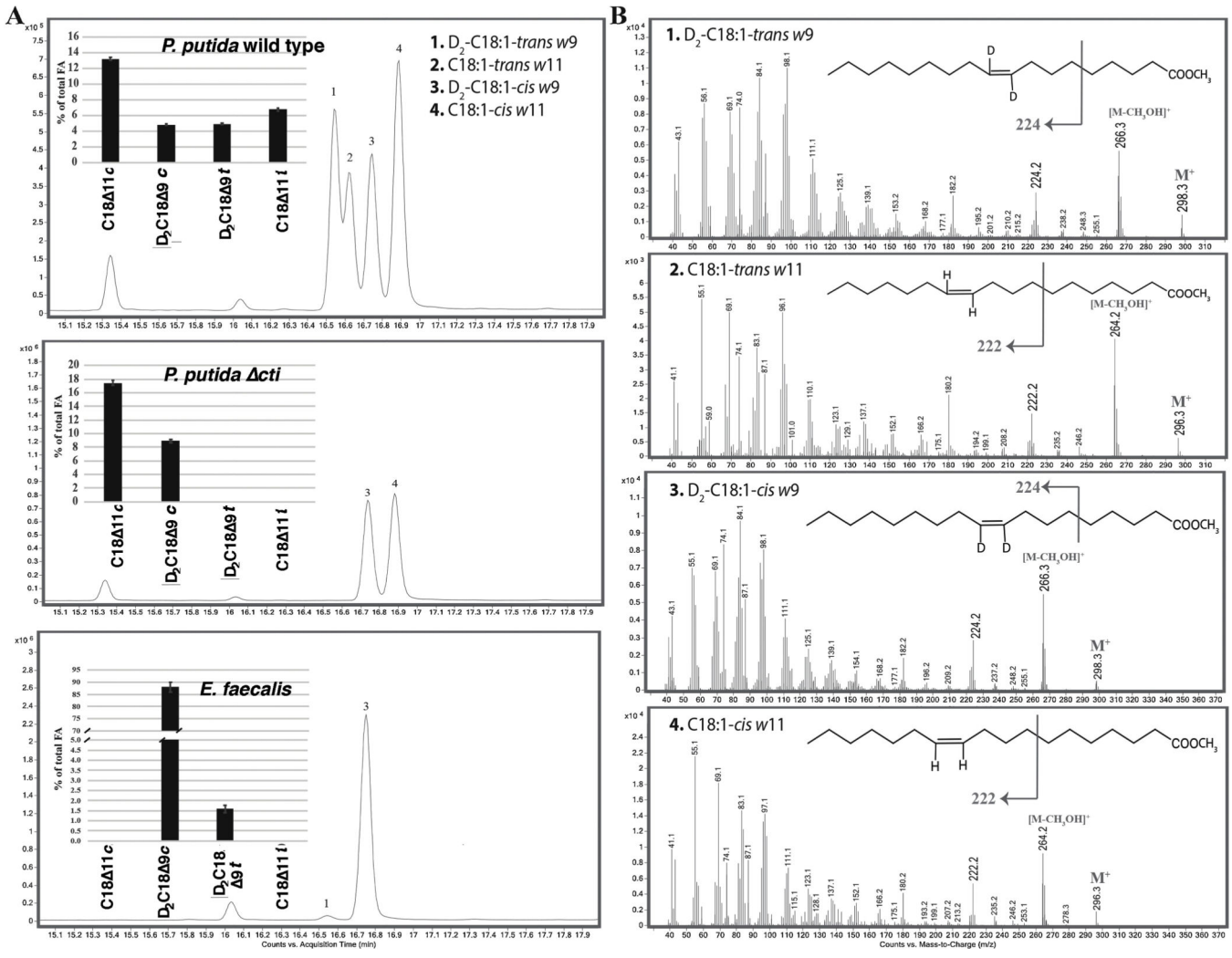

Figure 3. *E. faecalis* FA2–2 forms *trans*-UFA using a mechanism similar to that seen in *P. putida*

(**A**) Representative total ion chromatograms and peak assignment of bacterial FAME showing that *P. putida* F1 and *E. faecalis* FA2–2 form *trans*-UFA using a similar mechanism. Bacteria were grown in M17-FA free medium supplemented with 9,10-D_2-oleic acid for 6.5 h and FAME were obtained as described in Material and Methods. Note that the fatty acids extracted from *P. putida* F1 cultures (top panel) show the peaks corresponding to D_2-C18:1-*trans* Δ*9,* C18:1-*trans* Δ*11*, D_2-C18:1-*cis* Δ*9* and C18:1-*cis* Δ*11*. In contrast (middle panel) the P. *putida* F1 Δ*cti* strain was unable to synthetize *trans*-UFA and has only two peaks, corresponding to D_2-C18:1-*cis* Δ*9* and C18:1-*cis* Δ*11* fatty acids, whereas the *E. faecalis* FA2–2 Δ*fabI* strain (an oleate auxotroph) has two peaks corresponding to D_2-C18:1-*trans* Δ*9* and D_2-C18:1-*cis* Δ*9* fatty acids (bottom panel). The percentages of C18:1 in total bacterial FA are superimposed onto the chromatograms, N=3.

(**B**) Representative MS spectra showing the fragmentation patterns of C18:1 fatty acid species. The shift of 2 atomic mass units was observed for D_2-C18:1 and indicated no deuterium loss was observed during the isomerization reaction in *E. faecalis* FA2–2. Peak

numbers and identifications are shown, N=3. For all panels, N indicates biological replicates corresponding to independent experiments.

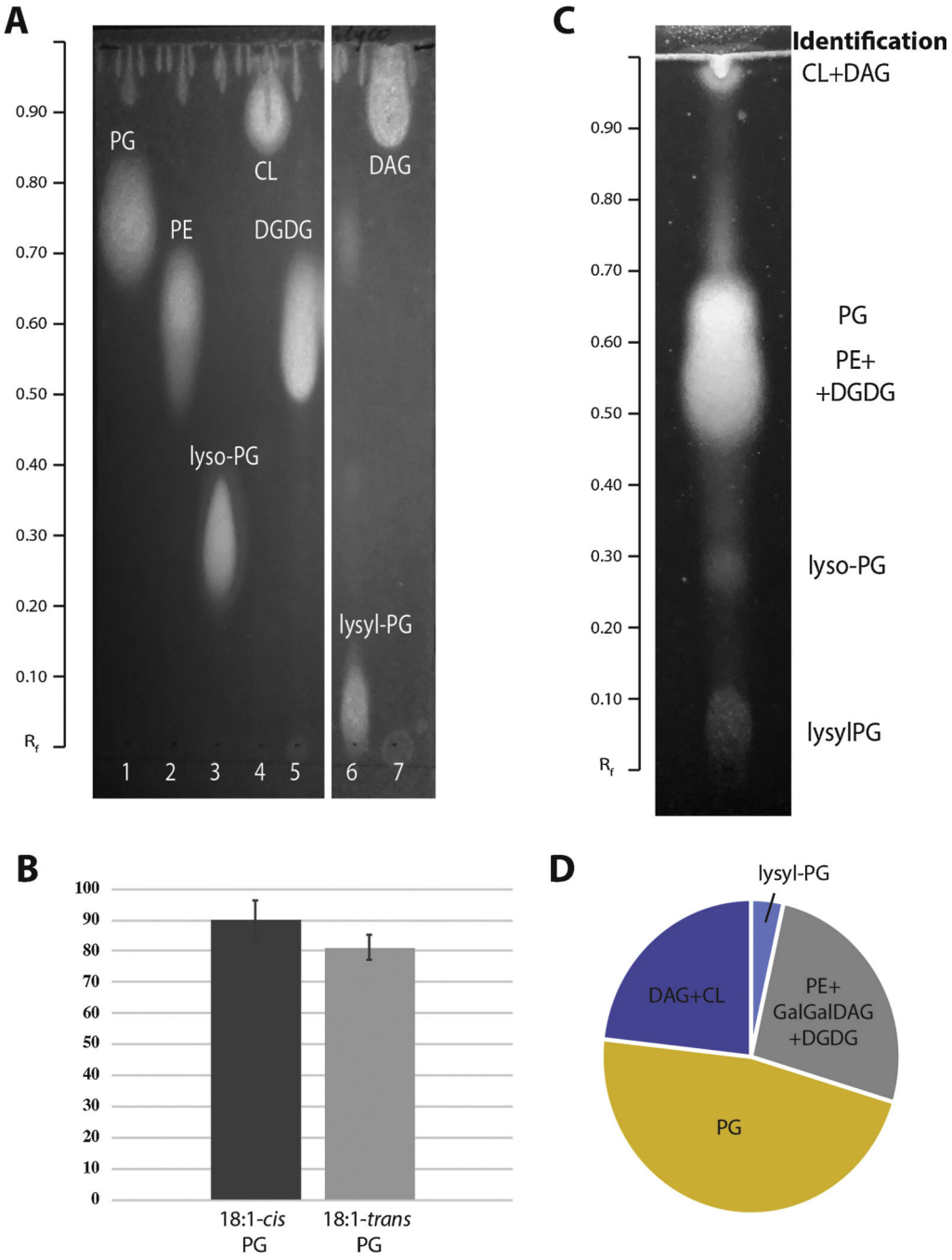

Figure 4. Distribution of *trans*-UFA in *E. faecalis* FA2–2 lipids

(**A**) Lipid standard profiles by HPTLC silica gel chromatography. Individual lipids were separated on HPTLC plates using $CHCl_3/CH_3OH/CH_3COOH$ (65/25/10, v/v/v) as solvent system and detected by UV fluorescence at 365 nm after spraying with a primulin dye solution.

(**B**) About 81% and 90% recovery of C18:1-*cis* and C18:1-*trans* fatty acids, respectively, from phosphatidylglycerol (PG) after separation on HPTLC plates. The PG-C18:1-*cis* and PG-C18:1-*trans* samples were first run on HPTLC plates. The lipid spots were scrapped off

and extracted by the Bligh and Dyer procedure. Esterification reaction was conducted as shown in material and methods. The recovery from HPTLC plate was calculated using the peak areas. The recovery in positive control sample (no HPTLC) was taken as 100%, and all other recoveries were expressed as percent of this value. Values shown are means ± SEM of three separate analyses.

(**C**) *E. faecalis* FA2–2 lipids separated and visualized on HPTLC plate as described for lipid standards (see Fig. 4A). The identification of individual HPTLC spots was made in comparison to control HPTLC plates of known standards.

(**D**) The content of *trans*-UFA in *E. faecalis* FA2–2 lipids showing that the bulk of *trans*-UFA was found in phosphatidylglycerol (PG) whereas no *trans*-UFA was found in lyso-PG. The *trans*-UFA content was assayed using GC MS as described in Fig. 4B and Materials and Methods. The negative control (silica gel from the used HPTLC plate outside of the lipid spots) was used to measure the noise rate. The peak areas obtained from the negative control samples were subtracted from the peak areas of lipid spots. The fractions of *trans*-UFA in each lipid spot were calculated using the peak areas. The total amount of *trans*-UFA was taken as 100%, and *trans*-UFA contents in lipid spots were expressed as percent of this value.

Designations for all panels, PG, phosphatidylglycerol; PE, phosphatidylethanolamine; lyso-PG, lyso-phosphatidylglycerol; CL, cardiolipin; DGDG, digalactosyldiacylglycerol; lysyl-PG, lysyl-phosphatidylglycerol; DAG, diacylglycerol; Rf, retention factor.

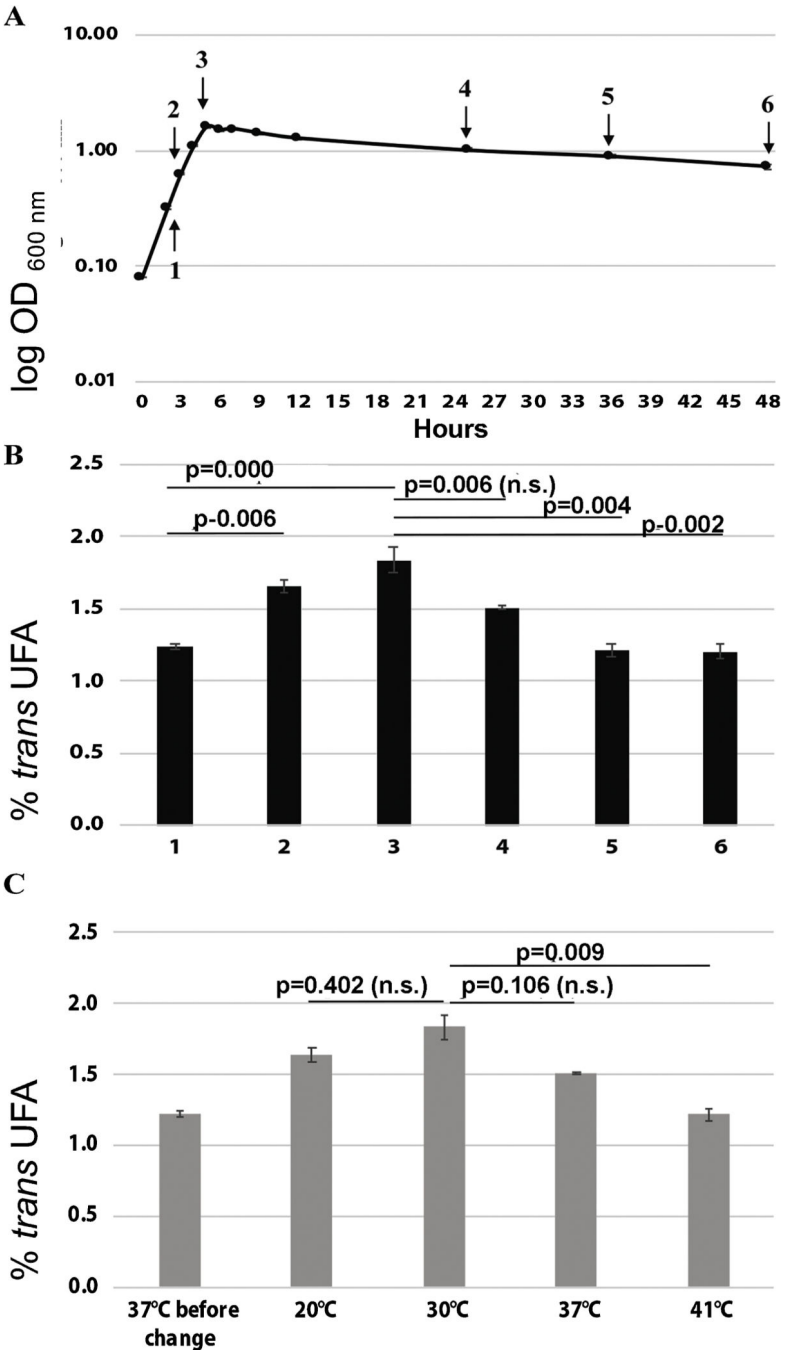

Figure 5. Effect of growth temperature and phase on production of *trans*-UFA in *E. faecalis* FA2–2

(**A**) Growth curve of *E. faecalis* FA2–2 at 37°C in M17 FA free medium. The arrows show the growth stages at which samples of cultures were taken for assays. N=3.

(**B**) Effect of growth phase on *trans*-UFA production in *E. faecalis* FA2–2. The numbers of the X axis show the growth stages at which samples of cultures were taken for GC MS analyses (see Fig. 4A). N=3.

(**C**) Effect of temperature shift on production of *trans*-UFA in *E. faecalis* FA2–2. Bacterial cultures grown in M17 FA free medium at 37°C with vigorous shaking (250 rpm) and constant aeration for OD_{600}=1.4 (corresponding to the point #3 on Fig. 4A) were incubated for 2 h at 20°C, 30°C, 37°C and 37°C. N=3. For all panels, mean ± SEM is shown. N indicates biological replicates corresponding to independent experiments. Statistical significance was determined by using one-way ANOVA (Turkey multiple comparison). n.s., not significant.

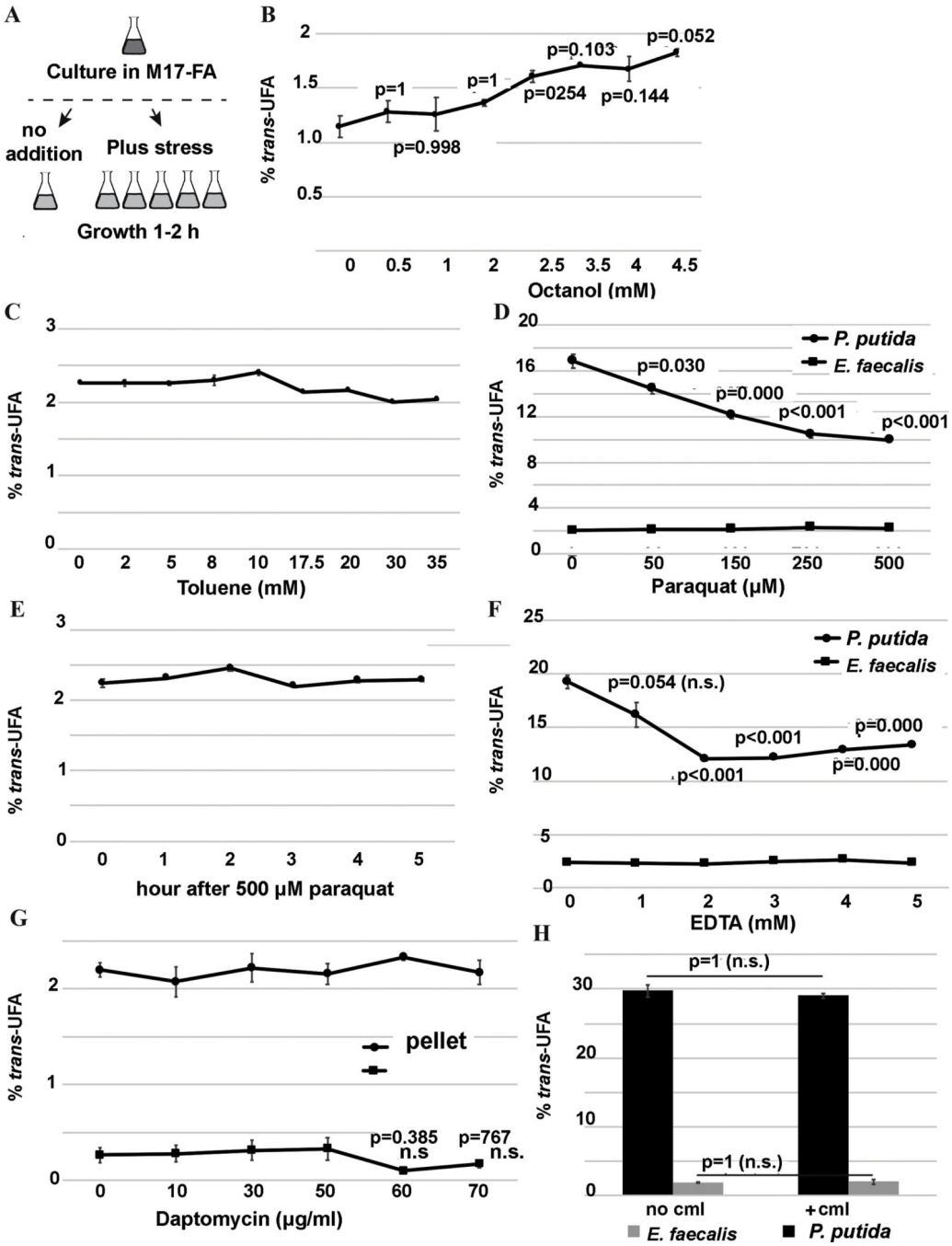

Figure 6. Addition of several stress inducing molecules does not modify *trans*-UFA production in *E. faecalis* FA2–2

(**A**) Workflow. Prior to treatment bacterial cultures were grown in M17 FA free at 37°C (for *E. faecalis* FA2–2) or at 30°C (for *P. putida* F1) with vigorous shaking (250 rpm) and constant aeration to the early stationary phase (OD_{600} of 1.4 and 5.5 for *E. faecalis* FA2–2 and *P. putida* F1, respectively). After treatment with different concentrations of stressors, bacterial samples were taken for GC MS assays. The viability of bacterial cultures after treatment was also monitored (see Fig. S2).

(**B**) Fractions of *trans*-UFA from total bacterial FA treated with 1-octanol. N=3.
(**C**) Fractions of *trans*-UFA from total bacterial FA treated with toluene. N=3.
(**D**) Fractions of *trans*-UFA from total bacterial FA treated with paraquat showing that in contrast to *P. putida* F1, production of *trans*-UFA in *E. faecalis* FA2–2 is independent on the oxidative stress caused by paraquat. N=3.
(**E**) Time-dependent fractions of *trans*-UFA from total bacterial FA treated with 500 μM paraquat showing that production of *trans*-UFA in *E. faecalis* FA2–2 is independent on the oxidative stress caused by paraquat. N=3.
(**F**) Fractions of *trans*-UFA from total bacterial FA treated with a metal chelator EDTA showing that in contrast to *P. putida* F1, production of *trans*-UFA in *E. faecalis* FA2–2 is independent on the EDTA addition. N=3.
(**G**) Fractions of *trans*-UFA from total bacterial FA treated with daptomycin. N=6.
(**H**) Fractions of *trans*-UFA from total bacterial FA treated with MIC concentrations of chloramphenicol (cml) N=3.

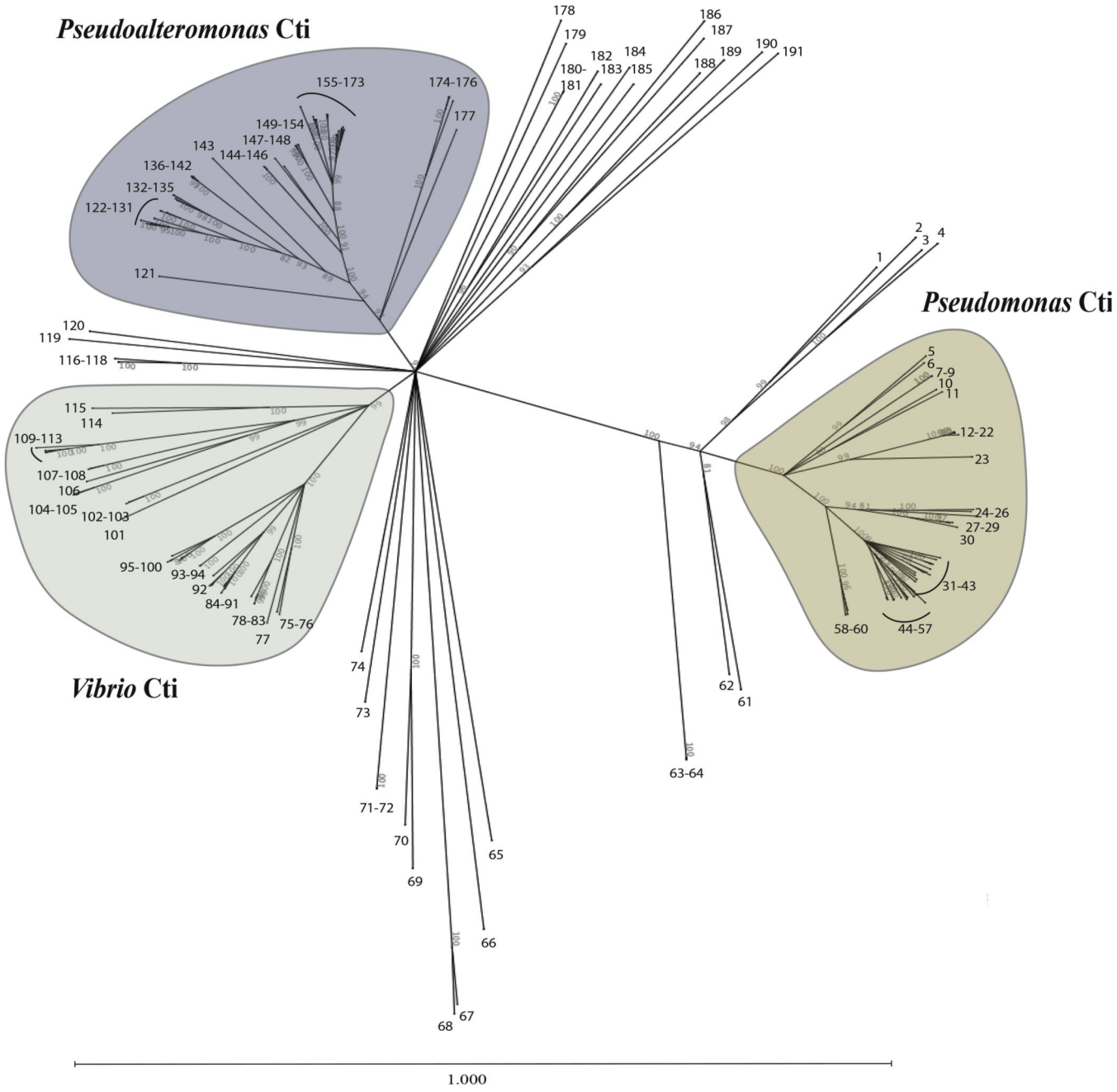

Figure 7. Neighbor-joining tree based on Cti protein sequences

Cti protein sequences were extracted from the MaGe Platform (http://www.genoscope.cns.fr/agc/mage). Node bootstrap values greater than 80% were used to construct the tree. The scale bar represents the average number of substitutions per site. Each number corresponds to one Cti sequence, whose reference and bacteria strains are reported in Table S3. Three major Cti clusters *Pseudomonas* Cti, *Vibrio* Cti and *Pseudoalteromonas* Cti are shown in orange, green and blue, respectively.

Table 1.

Detection of *trans*-UFA in *E. faecalis* FA2–2 cultures

Strain	**% of total FA**			
	***trans*-UFA**	**cyclo-FA**	***cis*-UFA**	**SFA**
E. faecalis FA2–2	1.6±0.0	14.8±0.9	39.7±1.1	43.9±0.2
E. coli K-12 MG1655	0.0±0.0	9.2±0.1	32.6±0.5	58.3±0.4
P. putida F1 WT	20.2±0.4	1.4±0.1	44.9±0.2	33.5±0.1
P. putida F1 Δ*cti*	0.0±0.0	4.8±0.1	53.1±0.7	42.1±0.6

Bacterial strains were grown in M17-FA free medium for 6.5 h. FAME samples were prepared according the standard protocol (Material and Methods). Mean ± SEM is shown. N=3. N indicates biological replicates corresponding to independent experiments. MG1655 is an *E. coli* wild type (WT) strain.