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Conjugation inhibitors compete with palmitic acid for binding to the conjugative traffic ATPase TrwD, providing a mechanism to inhibit bacterial conjugation

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Running Title: A novel drug-target to inhibit bacterial conjugation

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Abstract

Bacterial conjugation is a key mechanism by which bacteria acquire antibiotic resistance. Therefore, conjugation inhibitors (COINs) are promising compounds in the fight against the spread of antibiotic resistance genes among bacteria. Unsaturated fatty acids (uFAs) and alkynoic fatty acid derivatives, such as 2-hexadecanoic acid (2-HDA), were previously reported as being effective COINs. The traffic ATPase TrwD, a VirB11-homolog in plasmid R388, is the molecular target of these compounds, which likely affect binding of TrwD to bacterial membranes. In this work, we demonstrate that COINs are abundantly incorporated into *Escherichia coli* membranes, replacing palmitic acid as the major component of the membrane. We also show that TrwD binds palmitic acid, thus facilitating its interaction with the membrane. Our findings also suggest that COINs bind TrwD at a site that is otherwise occupied by palmitic acid. Accordingly, molecular docking predictions with palmitic acid indicated that it shares the same binding site as uFAs and 2-HDA, albeit it differs in the contacts involved in this interaction. We also identified 2-bromopalmitic acid, a palmitate analog that inhibits many membrane-associated enzymes, as a compound that effectively reduces TrwD ATPase activity and bacterial conjugation. Moreover, we demonstrate that 2-bromopalmitic and palmitic acids both compete for the same binding site in TrwD. Altogether, these detailed findings open up a new avenue in the search for effective synthetic inhibitors of bacterial conjugation, which may be pivotal for combating multidrug-resistant bacteria.

Introduction

Bacterial conjugation is one of the main mechanisms whereby bacteria become resistant to antibiotics (1). Therefore, there is a major interest in the search for conjugation inhibitors (COINs).

In conjugation, DNA is transferred between two bacterial cells through a type IV secretion system (T4SS), a multi-subunit complex encoded by the conjugative plasmid. Canonical T4SS consists of 11 proteins, named VirB1 to VirB11, after *Agrobaterium tumefaciens* T4SS (2,3). T4SS architecture is well preserved in most conjugative bacteria, consisting of four distinct sections: the pilus, the core channel complex, the inner membrane platform and the ATPases that provide the energy for substrate transport and pilus biogenesis (4). COINs have been proposed to target essential components of the T4SS (5). Promising results have been obtained with unsaturated fatty acids (uFAs), which specifically inhibit plasmid conjugation without inhibiting *Escherichia coli* growth (6,7). The traffic ATPase TrwD, the VirB11 homolog in plasmid R388, was shown to be the target for this inhibition by uFAs (8). TrwD contributes to pilus biogenesis and DNA translocation (9), thus working as a molecular switch between pilus synthesis and substrate transport (10). TrwD was specifically inhibited *in vitro* by the same uFAs and alkynoic fatty acid derivatives (2-aFAs) that inhibited bacterial conjugation *in vivo*, such as linoleic acid or 2-hexadecynoic acid (2-HDA), respectively. These compounds acted as non-competitive inhibitors, with no effect on the affinity of the protein for ATP or ADP substrates (8). In contrast, saturated fatty acids, such as palmitic acid, showed no inhibitory effect in conjugation experiments (6) and in ATPase assays (8).

TrwD belongs to the secretion ATPase superfamily, which also includes members of Type II secretion, Type IV pilus and flagellar biogenesis machineries (11). All members of this superfamily are hexameric ATPases, in which each monomer is formed by two domains at the N- and C- termini (NTD and CTD, respectively), connected by a flexible linker of variable length (11,12). ATPase catalysis is driven by swapping the NTD over the CTD, due to the flexibility of the linker (13,14). Blind docking predictions suggested a putative binding site for uFAs and 2-aFAs located at the end of the NTD and beginning of the linker region that connects it to the CTD, where the nucleotide binding site is located (8). These predictions are compatible with a model in which the mode of action of the inhibitors consisted in preventing the swapping movements between the N- and C- terminal domains that are required in the catalytic cycle of the protein. VirB11 ATPases interact with the cytoplasmic site of the membrane through its N-terminal domain (15,16). Therefore, it is likely that the inhibitory effect of uFAs and 2-aFAs occurs by affecting the membrane binding capability of TrwD. In this work, we demonstrate that the 2-alkynoic fatty acid 2-HDA incorporates into bacterial membranes, replacing palmitic acid as the major component of the membrane. We also show that TrwD binds palmitic acid, which suggests that uFAs and 2-AFAs act as inhibitors by binding TrwD at a site that is otherwise occupied by palmitic acid. Accordingly, docking predictions with palmitic acid indicate that it shares the same binding site as linoleic acid and 2-HDA.

Interestingly, we also discovered that 2-bromopalmitic acid (2-BP), a proven compound to block palmitate incorporation onto a variety of membrane-associated eukaryotic proteins (17), is also an inhibitor of TrwD ATPase activity and bacterial conjugation. Moreover, we demonstrate that 2-bromopalmitic and palmitic acids compete for the same binding site in TrwD, as shown by the increase in the value of the apparent inhibition constant in the presence of the saturated fatty acid. Recently, tanzawaic acids also appeared as a novel group of bacterial conjugation inhibitors (18). All these compounds share similar chemical characteristics: a carboxylic group, a long unsaturated aliphatic chain and the presence of double or triple bonds. The finding of 2-BP as an effective inhibitor allows us to conclude that inhibition does not occur by the presence of double or triple bonds in these compounds but by the conformation these fatty acids acquire upon TrwD binding. These new findings open a new avenue in the search of new and more effective synthetic inhibitors by structure-based drug design methods.

Results

2-Alkynoic fatty acids incorporate into bacterial membranes, where they exert an inhibitory effect on conjugation.

Unsaturated fatty acids (uFAs) and 2-alkynoic fatty acids specifically inhibit plasmid conjugation without inhibiting *E. coli* growth (6,7). Recently, we identified TrwD, the VirB11 homolog in plasmid R388, as their molecular target (8). As VirB11 traffic ATPases transiently interact with the cytoplasmic site of the membrane (19), 2-alkynoic fatty acids, such as 2-HDA, might be incorporated into the bacterial membrane to exert an inhibitory conjugation effect.

In an attempt to find out if fatty acid derivatives that act as inhibitors (COINs) do incorporate into membranes, *E. coli* cells were grown in the presence and absence of the inhibitor 2-HDA. After 18 h incubation, the composition in esterified fatty acids (FAMEs) from membrane phospholipids and free fatty acids was checked by Gas Chromatography-Mass Spectrometry (GC-MS). Results revealed that the phospholipid fatty acids present in untreated bacteria are primarily saturated, being palmitic acid (16:0) the most abundant (almost 50% of the total membrane phospholipids contained palmitic acid) (Figure 1). When cells were grown in the presence of 2-HDA (50 μg/ml), bacteria incorporated this exogenous fatty acid to their membranes. Moreover, the major component of the total phospholipids in treated bacteria was 2-HDA (44% w/w). The incorporation of 2-HDA into the bacterial membrane was accompanied by a 2-fold decrease of palmitic acid in the phospholipid fraction. In contrast, the percentage of palmitic acid in the free fatty acid fraction was not significantly altered when cells were treated with 2-HDA (Figure 1). As result of this experiment, we conclude that incorporation of 2-HDA into the bacterial membrane alters the utilization of endogenous fatty acids in the biosynthesis of phospholipids, with a significant amount of palmitic acid being replaced by 2-HDA in the bacterial membrane.

TrwD binds palmitic acid

The surprising effect observed in the previous experiment suggested that such a replacement might in turn affect the association of TrwD to the membrane. In contrast to uFAs or 2-HDA, saturated fatty acids neither inhibit bacterial conjugation (6), nor TrwD ATPase activity *in vitro* (8). Nonetheless, docking predictions with palmitic acid suggested that its binding site is the same as that for uFAs and 2-HDA, albeit adopting a different conformation due to the lack of double or triple bonds (8).

The molecular bases that underlie TrwD binding to the membrane are unknown. Therefore, we investigated a possible lipid-protein association via palmitic acid, by studying the binding of TrwD to ^{14}C-labeled palmitic acid. Purified TrwD protein was incubated with palmitic acid at different ratios, and samples were analyzed by polyacrylamide gel electrophoresis under non-denaturing conditions (Figure 2A). Radiolabeling of TrwD with palmitic acid was directly related to increasing palmitate/protein ratios. As control, TrwA protein (a conjugative protein that does not bind to the membrane) was incubated with ^{14}C-labeled palmitic acid at the highest palmitic acid/protein ratio. No radioactivity signal was observed for this control protein. Similar experiments, carried out with ^{14}C-labeled palmitoyl-CoA, gave identical results. In this latter case, it was possible to observe the unbound labeled palmitoyl-CoA running at the front of the gel (Figure 2B).

S-palmitoylation, a covalent attachment of fatty acids to cysteine residues of proteins, enhances the surface hydrophobicity and membrane affinity of proteins, playing an important role in modulating protein trafficking (20). TrwD contains five cysteine residues in its sequence, with one of them placed at the N-terminal domain (Cys44). Although this is a post-translational modification observed in eukaryotic proteins, we wanted to check if TrwD was covalently bound to palmitic acid. The association of labeled palmitate with TrwD was sensitive to denaturation induced by SDS gel electrophoresis, which suggests that the binding is non-covalent and there is no thioester bond involved in such association.

2-Bromopalmitic acid inhibits TrwD ATPase activity

2-Bromopalmitic acid (2-BP) is a palmitate analog that acts as inhibitor of many membrane-associated enzymes (17). It is an irreversible inhibitor that blocks palmitate incorporation onto

proteins. The exact mechanism responsible for 2-BP mediated inhibition is not known. It is thought to inhibit protein acyl-transferases (21), but recent reports showed that 2-BP is a non-selective probe, with many other different targets (22).

Thus, we analyzed the ATPase activity of TrwD in the presence of increasing concentrations of 2-BP (Figure 3). Analysis of the kinetics showed an inhibition pattern similar to 2-HDA or unsaturated fatty acids. In all cases, data did not fit to a Michaelis-Menten inhibition kinetic curve, but to a sigmoidal Hill equation, which suggested a cooperative effect in the inhibition kinetics. The apparent inhibition constant [Ki(app)] of 2-bromopalmitic acid was 21.5 ± 1.5 μM, similar to that obtained for 2-HDA [Ki(app)= 29.7±2.1μM] (Figure 3).

Previously discovered conjugation inhibitors (COINs), such as uFAs (oleic and linoleic acids), chemically synthesized 2-HDA and derivatives (7) or tanzawaic acids (18), shared similar chemical characteristics: a carboxylic group, a long unsaturated aliphatic chain and the presence of double or triple bonds. The finding that 2-BP is an effective inhibitor was at first surprising. Nevertheless, the results shown above allow us to conclude that inhibition does not occur by the presence of double or triple bonds in these compounds but, indirectly, by the conformation these fatty acids acquire when bound to TrwD. Docking predictions with 2-BP indicate that the binding site for the palmitic acid analog is also in the same region as linoleic acid, 2-HDA and palmitic acid (Figure 4). In this case, the presence of a halogen group (2-bromo) within the saturated fatty acid can result in a similar inhibitory effect, but modifying the mode of binding.

2-bromopalmitic and palmitic acids compete for the same binding site in TrwD

To determine if 2-bromopalmitic and palmitic acids compete for the same binding site, we conducted a further characterization of the mechanism of inhibition. ATP turnover was measured at increasing concentrations of 2-BP in the presence of palmitic acid (500 μM) (Figure 5). Under these experimental conditions, the apparent inhibition constant [Ki(app)] of 2-BP increased 3-fold (from 21.5 ± 1.5 μM to 65.4 ± 1.4 μM) (Figure 3), suggesting that palmitic acid and 2-BP compete for the same binding site in TrwD.

2-Bromopalmitic acid inhibits R388 conjugation

In a previous work (8), we observed that 2-alkynoic fatty acids that inhibited bacterial conjugation, such as 2-hexadecynoic acid (2-HDA), 2-octadecynoic acid (2-ODA) or 2,6-hexadecadiynoic acid (2,6-HDA), also inhibited TrwD ATPase activity *in vitro*. In contrast, those with no effect *in vivo*, such as alcohol or tetrahydropyranyl-ether derivatives, did not affect the *in vitro* TrwD ATPase activity either. Therefore, once we discovered that 2-BP was an effective inhibitor of TrwD ATPase activity, we checked if this compound was also an inhibitor of bacterial conjugation. R388 conjugation frequencies at increased concentrations of 2-BP are shown in Figure 6.

Bacterial conjugation was monitored by a high-throughput assay based on fluorescence emission by transconjugant cells. Control assays were carried out to discard any effect on bacterial growth or plasmid stability. Addition of 2-BP (0.3 mM) resulted in the inhibition of R388 conjugation (Figure 6). 2-BP inhibited R388 conjugation to levels similar to those obtained with 2-HDA (to about 2 % of the control without COIN). Moreover, when palmitic acid (500 μM) was added to the conjugation media, a higher concentration of 2-BP was required to reach a similar inhibitory effect. This result perfectly correlates with that obtained *in vitro*, on TrwD ATPase activity, and confirms 2-BP as an effective COIN.

Discussion

VirB11 proteins belong to a large family of hexameric AAA+ traffic ATPases, which includes proteins involved in Type II secretion and in Type IV pilus and flagellar biogenesis (11). VirB11 from *Agrobacterium tumefaciens* localized at the cell pole, as shown by epifluorescence microscopy of a GFP-VirB11 fusion protein (23). The protein required neither ATP nor other VirB proteins for its polar localization (19). In contrast, in the presence of a

complete T4SS machinery, VirB11 localized all over the bacterial cell perimeter (24). Accordingly, *in vitro* experiments had previously shown that TrwD, the VirB11 homolog in plasmid R388, interacts with phospholipids in lipid vesicles, causing lipid vesicle aggregation and intervesicular lipid mixing, supporting the hypothesis that TrwD also behaves as a membrane-associated protein (16). Therefore, although it is proved that the protein localizes in the bacterial inner membrane, the molecular bases underlying this interaction with membrane lipids were unknown.

Recently, we identified TrwD as the molecular target for fatty acid-mediated inhibition of conjugation (8), showing that unsaturated fatty acids (uFAs) and 2-alkynoic fatty acids (aFAs) that inhibited bacterial conjugation, such as 2-HDA, 2-ODA or 2,6-HDA, also inhibited TrwD ATPase activity. In contrast, saturated fatty acids such as palmitic acid, neither showed an inhibitory effect in conjugation experiments nor in ATPase assays. In an attempt to explain how these compounds can act as inhibitors of bacterial conjugation, we investigated the putative interaction of uFAs and aFAs with the bacterial membrane. Indeed, our results revealed that the phospholipid fatty acids present in untreated bacteria are mainly saturated, being palmitic acid the most abundant. However, when cells were grown in the presence of 2-HDA, this compound was incorporated into the membrane and became the major component of the total phospholipid fraction (Figure 1). More importantly, a significant amount of palmitic acid was replaced by 2-HDA in the membrane. Therefore, it was reasonable to think that this replacement of palmitic acid by fatty acids with double or triple bonds might be responsible of such an inhibitory effect. Thus, we wondered whether TrwD interaction with the membrane was occurring via palmitic acid under normal growth conditions. Supporting this idea, a blind docking search of the palmitic acid in TrwD structure revealed the same binding site as for uFAs and 2-HDA, albeit it differs in the contacts involved in the interaction (8). To confirm the putative binding of saturated fatty acids, purified TrwD protein was incubated with ^{14}C-labeled palmitic acid and the resulting complex was analyzed in native gels (Figure 2). Radiolabeling of TrwD with palmitic acid was directly related to increasing palmitate/protein ratios.

A wide variety of proteins associate to the membrane by a covalent attachment to palmitic acid (20). In these palmitoylated proteins, the fatty acid is bound to a cysteine residue through a thioester linkage. This is a reversible process that allows a transient binding to the membrane, which is crucial in many biological processes. This lipid modification is common in eukaryotic cells (20) but is poorly documented in prokaryotes. To our knowledge, there is only one example of *S*-palmitoylation for a bacterial protein (25). TrwD presents five cysteine residues. One of them is placed at the NTD, so palmitoylation might be a possible mechanism of interaction of TrwD with the membrane. Therefore, we checked if the association of radio-labeled palmitate with TrwD was resistant to denaturation induced by SDS gel electrophoresis. No signal of radiolabeled protein was observed in these gels. Samples of purified protein incubated with palmitic acid were also analyzed by mass spectrometry, but no difference in molecular mass was observed when the sample was compared to a control in the absence of the fatty acid. Therefore, our data indicate that the binding to palmitic acid is non-covalent, excluding the possibility that the association of palmitate with TrwD is due to protein palmitoylation. In the literature, there are also several examples of proteins that associate to the membrane via a non-covalent binding to saturated fatty acids, such as Toll-like receptors (TLRs) (26). In this case, the polyunsaturated fatty acid binds to a lipid binding pocket in the protein receptor. Intriguingly, TLRs are also inhibited by polyunsaturated fatty acids (27), as in the case of TrwD.

To study the binding of palmitic acid to TrwD, we explored the binding capacity of an analog, namely 2-bromopalmitic acid (2-BP). This fatty acid was found to act as inhibitor of many membrane-associated enzymes that bind palmitic acid (17). First, we checked if this palmitate analog also inhibited TrwD activity. We analyzed the ATPase activity of the protein in the presence of increasing concentrations of 2-BP (Figure 3). The inhibition pattern was similar to that obtained

with 2-HDA or uFAs, with an apparent inhibition constant similar to that obtained for 2-HDA. In a previous work, we found that the same fatty acid derivatives that were effective inhibitors of bacterial conjugation were also capable of inhibiting TrwD ATPase activity, and *vice versa*, those unable to inhibit conjugation also failed to inhibit TrwD activity, with a perfect correlation between the *in vivo* and *in vitro* data (8). In the same way, in this work we demonstrate that 2-BP inhibited R388 conjugation, as in the case of 2-HDA (Figure 6). Moreover, when palmitic acid was added to the conjugation media, a higher concentration of 2-BP was required to reach a similar inhibitory effect. This effect is probably due to the incorporation of 2-BP into the bacterial membrane and the consequent replacement of palmitic acid by 2-BP, an effect that has already been observed for 2-HDA (Figure 1). Therefore, these inhibitors seem to act by displacing the more abundant saturated acid in the membrane.

In an attempt to determine if 2-BP and palmitic acid compete for the same binding site in TrwD, ATP turnover was measured at increasing concentrations of 2-BP in the presence of palmitic acid (Figure 5). The apparent inhibition constant (Ki(app)) of 2-BP raised from 21.5 ± 1.5 μM to 65.4 ± 1.4 μM (Figure 3), which suggests that both fatty acids (palmitic acid and 2-BP) compete for the same binding site in TrwD. Although it is generally accepted that 2-BP blocks S-palmitoylation by inhibiting protein acyl transferases (21), recent work has shown that 2-BP has many other targets, leading the authors to suggest that the compound might exert the inhibition by direct competition with palmitic acid (22). The results obtained with TrwD in this work agree with this hypothesis and might be extensive to proteins that associate with the inner membrane via interaction with palmitic acid.

In summary, in this work we describe 2-BP, a palmitate analog, as a new inhibitor of bacterial conjugation. As previously described inhibitors, which are also fatty acid derivatives, 2-BP acts on the traffic ATPase TrwD as its molecular target. The results presented here demonstrate that inhibitors such as 2-HDA, incorporate into the bacterial membrane and replace a significant fraction of palmitic acid. Although it was known that VirB11 proteins localize in the bacterial inner membrane, the molecular bases of this interaction had not been identified. We have proven that TrwD interacts with palmitic acid, which suggests TrwD interacts with the membrane by binding this fatty acid. This work shows that COINs act by competing with palmitic acid for the same binding site in TrwD. Moreover, fatty acid derivatives lacking the carboxylic group do not inhibit the protein but are still predicted to bind to the same pocket, albeit with different protein contacts (Ripoll-Rozada, et al, 2016), reinforcing the importance of the stereospecificity of the binding in order to exert the inhibition. These differences in the mode of binding might explain the different behavior of palmitic acid and the fatty acid derivatives that act as inhibitors, such as 2-BP (Figure 4). In the latter case, the bromine substituent induces a different conformation in the carboxylic group, which is forced to move 120 degrees apart, creating closer contacts with basic residues of the flexible linker. This linker plays an essential role in ATP catalysis, allowing the movement of the N-terminal domain (NTD) over the C-terminal domain (CTD) (13,14). In contrast, binding predictions for palmitic acid locate its carboxylic group in the opposite direction, out of the linker region. These differences might explain why the presence of the bromide ion or double and triple bonds in the fatty acid derivatives that act as inhibitors affect the catalytic mechanism of TrwD.

It is worth noting that uFAs and 2-aFAs are effective inhibitors of IncW plasmids such as R388, but other plasmid groups, such as IncN and IncP, are not affected by them (6,7). In contrast to TrwD, TrbB, its homolog in RP4 plasmid contains a much shorter linker, being more similar to HP0525 (*H. pylori*) than to VirB11 (Ripoll-Rozada, 2013). Likewise to HP0525, TrbB does not have a pocket between the linker and the NTD, and therefore, it is not surprising to observe a lack of effect of COINs in this system. By contrast, VirB11 homologs from IncN plasmids are close TrwD relatives. However, plasmids from this group, such as pKM101, are not inhibited by these COINs, either. A close inspection of TraG structure, the VirB11 homolog in plasmid pKM101, revealed substantial differences with TrwD. The equivalent binding site in TraG

presents a different global charge balance and is occluded by two hydrophobic residues, which gives no room for fatty acid binding (Figure 1S). Moreover, docking predictions in TraG with 2-BP and 2-HDA indicate that the binding site for these fatty acids is in a different region (Figure 2S). This binding site is also at the NTD, but it does not involve the linker and, therefore, it does not affect the catalytic mechanism of the enzyme, which might explain the lack of inhibition of TraG by these fatty acid derivatives. These new findings will allow us to optimize the chemical structure of these compounds by structure-based drug design methods, in order to design new and more effective derivatives that act as COINs.

Experimental procedures

***Proteins and fatty acids*.** Cloning, overexpression and purification of TrwD and TrwA proteins were carried out as described previously (28,29). Palmitic and 2-bromopalmitic acids were purchased from Sigma-Aldrich. Palmitic acid derivatives, such as [^{14}C(U)] and palmitoyl-Coenzyme A, [Palmitoyl-1-^{14}C] were purchased from PerkinElmer. 2-HDA was synthesized as previously described (30).

Gas Chromatography-Mass Spectrometry (GC-MS). *E. coli* ATCC 25922 (American Type Culture Collection, Manassas, VA) was grown in Luria-Bertani broth (LB, Lennox, Fisher Scientific, Fair Lawn, NJ) medium in the presence and absence of 2-HDA (50 µg/mL) at 37˚C for 18 h. Then, cells were collected, extracted by using 15 mL of chloroform:methanol (2:1, v/v), shaken (Labnet, Edison, NJ) for 30 min at 200 rpm, and sonicated into Ultrasonic Cleaner (Fisher Brand FB11201, Germany) at 37 kHz (100 % power, 390 W) for 15 min at room temperature. Phospholipids and free fatty acids were separated from total lipids by using silica gel column chromatography. Free fatty acids were eluted by adding 15 mL of acetone, while phospholipids were eluted by adding 15 mL of hexane. Solvents from both acetone and hexane fractions were removed by rotoevaporation (Büchi, Rotavapor R-114, Switzerland) and the resulting lipid content was treated with 3 mL of methanol and 150 µl of 12 M HCl and subsequently refluxed for 3 h. Once the transesterification reaction was completed, methanol was removed by rotoevaporation and fatty acid methyl esters (FAMEs) were analyzed by using GC-MS (Hewlett Packard Series II MS ChemsStation coupled to an Hewlett Packard 5972 Series Mass Selective Detector equipped with a 30 m x 0.25 mm special performance capillary column HP-5MS of polymethyl siloxane crosslinked with 5 % phenyl methylpolysiloxane). The selected temperature method was: 120 °C initial temperature, 5 °C/min temperature rate, and 260 °C final temperature. The split ratio was set at 10:1 for all analyses. All fatty acids were identified based on their molecular ion, base peak, fragmentation pattern and retention time and then expressed as a percentage of total FAs.

***Radio-labeling assays*.** Purified proteins were incubated with ^{14}C-labeled fatty acids for 10 min at room temperature. Then, samples were analyzed by native polyacrylamide gel electrophoresis (Native-PAGE), following the protocol previously described (31). 4.5 % polyacrylamide gels were run at pH 8.5 for 140 min in an ice bath, with a constant amperage (30 mA). Then, gels were dried, exposed overnight and visualized using a Fluoro Image Analyzer FLA-5100 (FujiFilm) to detect radiolabeled proteins on electrophoretic bands.

***ATP hydrolysis assays*.** Steady-state ATP hydrolysis activity *in vitro* was measured with the EnzCheck™ Kit (Invitrogen) in a UV-1800 spectrophotometer (Shimadzu), as described previously (28). Inorganic phosphate (Pi) released after ATP hydrolysis was monitored as an increase of absorption at 360 nm for 10 min following manufacturer's instructions and components: 0.2 mM 2-amino-6-mercapto-7-methylpurine riboside (MESG) and 1 unit/ml of purine nucleoside phosphorylase (PNP). Fatty acids diluted in DMSO were added to the reaction buffer, consisting of 50 mM Tris-HCl pH 8.5, 75 mM potassium acetate, 10 µM magnesium acetate, 1 mM ATP and 10 % glycerol (w/v). Reactions were started by the addition of the ATPase TrwD (2 µM).

***Conjugation assays*.** Conjugation frequencies (CF) were obtained with a fluorescence-based assay previously described (7). Strain DH5α carrying the conjugative plasmid pJC01, which expresses GFP under the control of T7 promoter,

was used as a donor. A streptomycin-resistant derivative of *E. coli* BL21 (DE3), expressing T7 RNA polymerase from the inserted phage, was used as recipient strain. Both strains at stationary phase were concentrated 4-fold and mixed at a 1:1 ratio. Mixture samples (10 µl) were spotted onto 96-well microtiter plates (Bioster a.s.) containing 150 µl LB, 1 % agar, 1 mM IPTG and different concentrations of fatty acids, with the help of a Biomek 3000 liquid-handling robot (Beckman Coulter). After incubation at 37 ºC for 6 h to allow conjugation, cells were resuspended in Phosphate-Buffered Saline (PBS) and transferred to a new plate. The optical density at 600 nm (OD_{600}) and GFP emission were measured in a Victor3 multi-label counter (PerkinElmer). CF was calculated as the ratio of absolute fluorescence emitted by transconjugant cells and the total number of cells (OD_{600}). Relative CF in the presence of a compound was determined as a fraction of the CF observed in controls performed in the absence of the inhibitor. To reproduce the conditions, equivalent volumes of solvent (DMSO) were added to control samples.

Molecular modelling and ligand docking. An atomic model of TrwD was generated by molecular threading using as template the atomic coordinates of *B. suis* VirB11 (2gza.pdb) (14), as previously described (28). The structural coordinates of palmitic acid, 2-HDA and 2-BP acid were retrieved from PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and prepared for docking as previously described (8). Files containing the atomic coordinates of the TrwD model and the fatty acids were submitted to the SwissDock server (http://www.swissdock.ch/) to run blind docking. Results were examined with UCSF Chimera. Binding poses with the best Full-Fitness (FF) score and minimal energy were finally selected.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article

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Figure 1

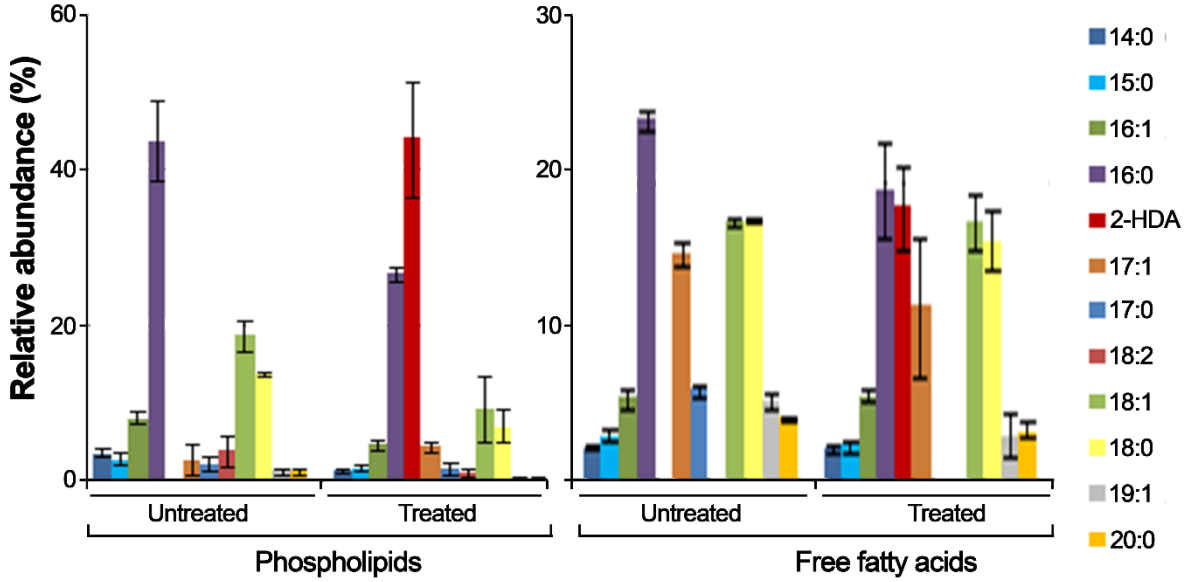

Figure 1. Composition of phospholipid fatty acids and free fatty acids in *E. coli* after exposure to 2-HDA. *E. coli* cells were grown at 37 °C for 18 h in Luria Broth (LB) containing 50 µg/mL 2-HDA (treated), or in its absence (untreated). Fatty acids were eluted, converted to methyl-esters and analyzed by Gas Chromatography – Mass Spectrometry (GC-MS), as described in materials and methods. Bars represent the relative abundance of different fatty acids obtained from three separated experiments (mean ± SD). Fatty acid nomenclature indicates the number of carbons followed by the number of double bonds. 2-HDA refers to 2-hexadecynoic acid.

Figure 2

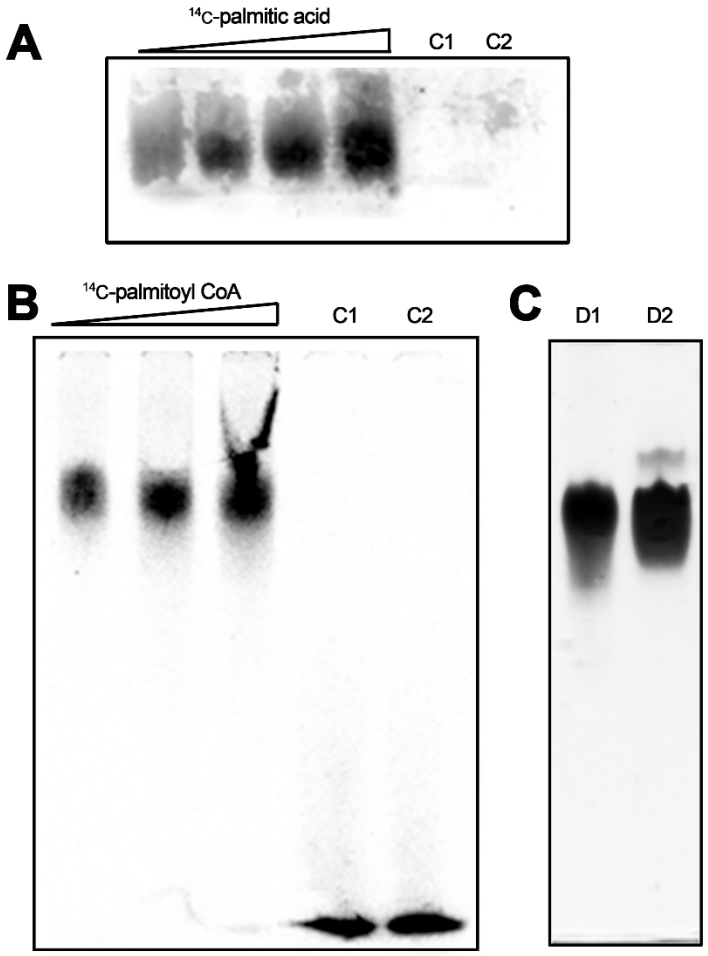

Figure 2. **TrwD binding to ^{14}C-labeled palmitic acid and palmitoyl-CoA.** (A). TrwD (80 µM) was incubated at increasing ^{14}C-labeled palmitic acid ratios (1:0.25, 1:0.5, 1:0.75 and 1:1 protein:palmitic acid molar ratios, respectively). Lane C1 corresponds to TrwD (80 µM) in the absence of fatty acids. Lane C2 is also a control experiment in which an alien protein, TrwA (80 µM), was incubated with ^{14}C-palmitic acid at a 1:1 protein:palmitic acid molar ratio. (B) TrwD (80 µM) was incubated at increasing ^{14}C-labeled palmitoyl-CoA ratios (1:0.5, 1:0.75 and 1:1 protein: palmitoyl CoA molar ratios, respectively). Lane C1 corresponds to ^{14}C-palmitoyl CoA (80 µM) in the absence of any protein. Lane C2, as in panel A, corresponds to TrwA (80 µM) incubated with ^{14}C-palmitoyl CoA (80 µM). Lane C1 and C2 radioactivity signals at the front of the gel correspond to free ^{14}C-labeled palmitoyl CoA. In both cases, protein-fatty acid complexes were analyzed by Native-PAGE and radiolabeled images were obtained after overnight exposure, as described in materials and methods. (C) TrwD (80 µM; Lane D1) and TrwA (80 µM; Lane D2) were analyzed by Coomassie Brilliant Blue-stained Native-PAGE, as described in materials and methods.

Figure 3

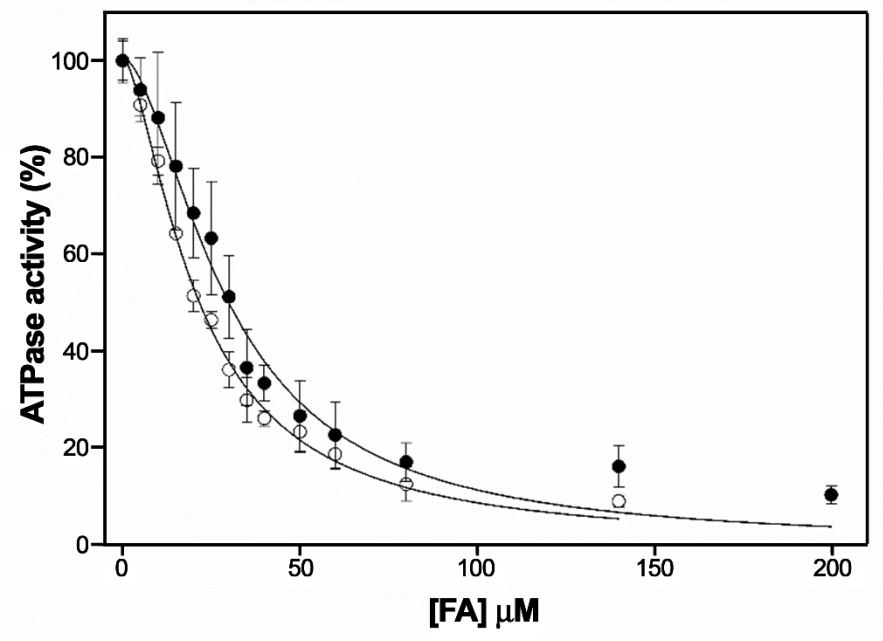

Figure 3. Determination of the kinetic parameters of inhibition by 2-bromopalmitic acid. ATP hydrolysis by TrwD (2 µM) was measured at increasing concentrations of 2-bromopalmitic acid (white circles) and compared with the values obtained at increasing concentrations of 2-HDA (black circles) (8). Data were fitted to a Hill inhibition equation (error bars: SD).

Figure 4

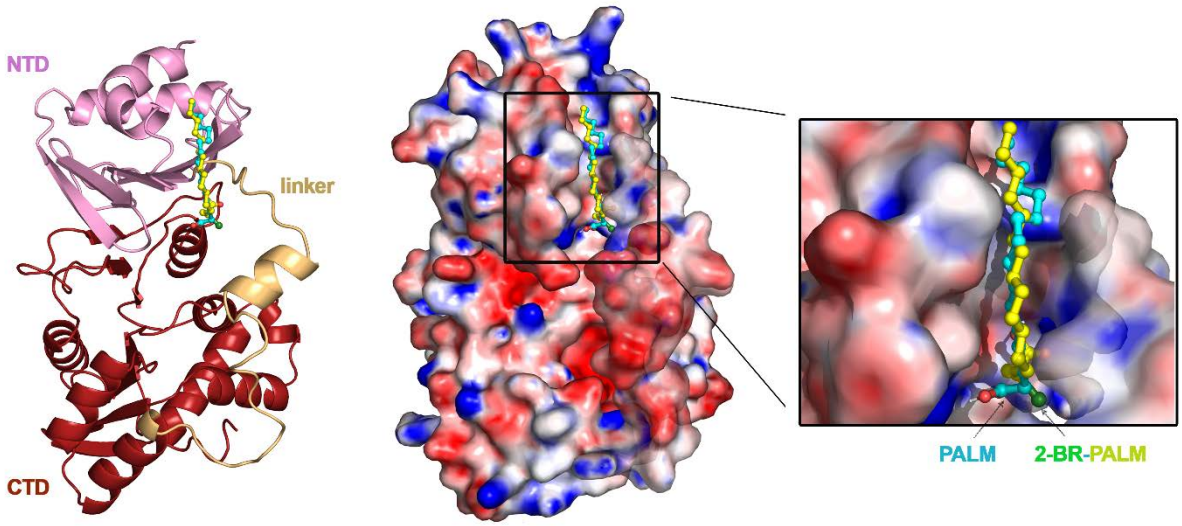

Figure 4. Blind docking of fatty acids into the molecular model of TrwD. Blind docking predictions between a molecular model of monomeric TrwD and fatty acid ligands (palmitic and 2-bromopalmitic acid) were performed using the EADock dihedral spacing sampling engine of the Swiss-dock server (32). Both fatty acids fit into a pocket located at the interface between the N-terminal domain (NTD, *pink*) and the linker region (*light orange*), which connects the NTD with the catalytic C-terminal domain (CTD, *brown*). Upper left and right panels correspond to the same view in cartoon and surface representation, respectively. The carbon chains of both fatty acids have a similar orientation (colors blue and yellow for palmitic and 2-bromopalmitic acids, respectively). However, the carboxylic group of the 2-bromo derivative is buried inside a pocket formed by the NTD and the linker domain of the protein, being the bromine atom (*dark green*) stabilized by electrostatic basic residues located in the region. On the contrary, the carboxylic group of the palmitic acid (red) is 120 degrees apart, out of the linker region. The panel on the right shows a zoom of the binding pocket, in which the basic residues surrounding the carboxylic groups of the fatty acids are better appreciated. For more clarity see also movie 1S in *Supporting information*.

Figure 5

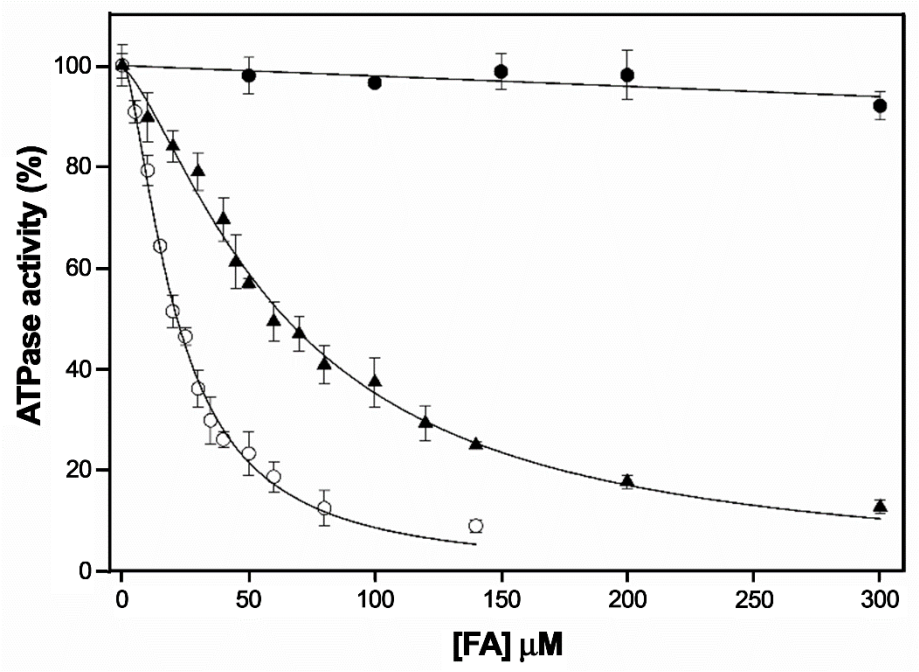

Figure 5. **2-Bromopalmitic and palmitic acids compete for the same TrwD binding site**. TrwD ATPase rates (2 µM) were measured as described in materials and methods, at increasing concentrations of palmitic acid (*dark circles*), 2-bromopalmitic acid (*white circles*) and mixtures of both fatty acids consisting of a fixed concentration of palmitic acid (500 µM) and increasing concentrations of 2-bromopalmitate (*dark triangles*). In the latter case, protein sample was first incubated with palmitic acid for 5 min, and then 2-bromopalmitic acid was added at the indicated concentrations. Data were fitted to a sigmoidal Hill equation for inhibition (error bars: SD).

Figure 6

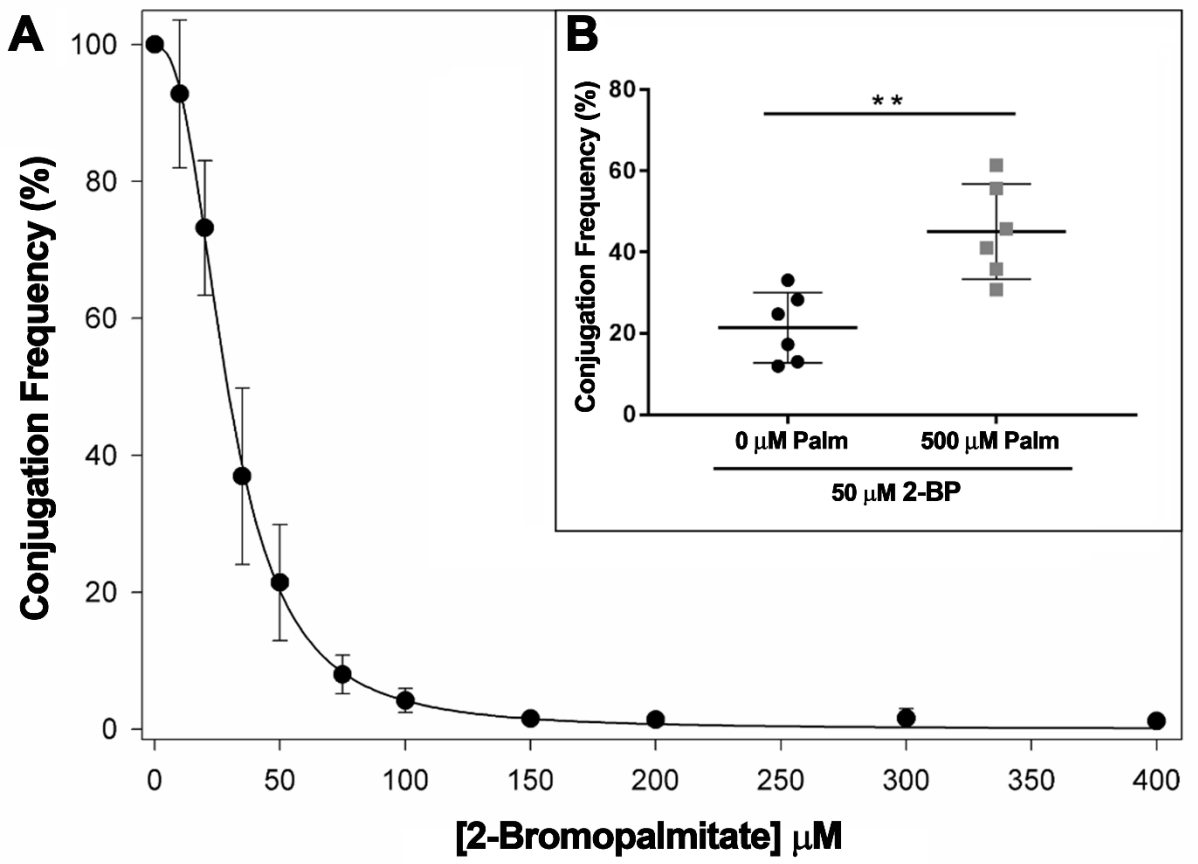

Figure 6. R388 conjugation frequency (CF) in the presence of increasing concentrations of 2-bromopalmitic acid. Bacterial conjugation experiments were performed by a fluorescence-based assay as described in materials and methods. A) Values represent the mean CF ± SD from at least four independent experiments, relative to a positive control in the absence of inhibitor (100 %). Panel B (inset figure) shows the relative CF from individual experiments in the presence of 2-bromopalmitic acid (50 μM) (*black circles*) and a mixture of both palmitic (500 μM) and 2-bromopalmitic acids (50 μM) (*grey squares*). Horizontal and vertical bars represent the mean ± SD of each group of data. Statistical significance analyzed by Student's t-test (**$p < 0.05$).

Conjugation inhibitors compete with palmitic acid for binding to the conjugative traffic ATPase TrwD, providing a mechanism to inhibit bacterial conjugation

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