Microbiology (2005), **151**, 3517–3526 DOI 10.1099/mic.0.28216-0

Unsaturated fatty acids are inhibitors of bacterial conjugation

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This report describes a high-throughput assay to identify substances that reduce the frequency of conjugation in Gram-negative bacteria. Bacterial conjugation is largely responsible for the spread of multiple antibiotic resistances in human pathogens. Conjugation inhibitors may provide a means to control the spread of antibiotic resistance. An automated conjugation assay was developed that used plasmid R388 and a laboratory strain of *Escherichia coli* as a model system, and bioluminescence as a reporter for conjugation activity. Frequencies of conjugation could be measured continuously in real time by the amount of light produced, and thus the effects of inhibitory compounds could be determined quantitatively. A control assay, run in parallel, allowed elimination of compounds affecting cell growth, plasmid stability or gene expression. The automated conjugation assay was used to screen a database of more than 12 000 microbial extracts known to contain a wide variety of bioactive compounds (the NatChem library). The initial hit rate was 1·4 %. From these, 48 extracts containing active compounds and representing a variety of organisms and extraction conditions were subjected to fractionation (24 fractions per extract). The 52 most active fractions were subjected to a secondary analysis to determine the range of plasmid inhibition. Plasmids R388, R1 and RP4 were used as representatives of a variety of plasmid transfer systems. Only one fraction (of complex composition) affected transfer of all three plasmids, while four other fractions were active against two of them. Two separate compounds were identified from these fractions: linoleic acid and dehydrocrepenynic acid. Downstream analysis showed that the chemical class of unsaturated fatty acids act as true inhibitors of conjugation.

Received 24 May 2005
Revised 11 July 2005
Accepted 20 July 2005

INTRODUCTION

Bacterial conjugation is an important adaptive mechanism that allows bacteria to exchange genetic information. Horizontal transfer of genes that confer selective advantages allows micro-organisms to respond quickly to a changing environment (de la Cruz & Davies, 2000; Koonin *et al.*, 2001). The extensive use of antibiotics exerts a heavy pressure on non-pathogenic populations of bacteria that live in contact with man. The resulting resistance genes are transferred to pathogenic species. As a result, antibiotic resistances are commonly found in human pathogens, and currently represent a serious problem in many infectious diseases (Mazel & Davies, 1999).

Previous studies demonstrated the potential for inhibiting bacterial conjugation by the addition of chemical compounds. Ou & Reim (1976) studied the inhibitory effect of 1,10-phenanthroline, a zinc-chelating agent, on F plasmid transfer. Michel-Briand & Laporte (1985) showed the inhibitory effect of nitrofurans in conjugation of plasmids belonging to six different incompatibility groups. However, nitrofurans cause a general disruption of bacterial DNA, and thus behaved as non-specific inhibitors. Mandi & Molnar

Abbreviation: DHCA, dehydrocrepenynic acid.

A detailed report of the nuclear magnetic resonance assays is available as supplementary data with the online version of the paper.

(1981) demonstrated that the major tranquillizer chlorpromazine is able to inhibit conjugal transfer of plasmid F. Chlorpromazine is a cationic amphipathic molecule that, by insertion into the inner leaflet of the membrane lipid bilayer, induces modifications of the membrane topology. It also produces a general stress response (Conter *et al.*, 2002) and reduces growth, so its inhibitory effect might have been caused by a general disturbance of cell physiology. Nalidixic acid or coumermycin inhibit conjugal transfer of plasmids F and R64 (Hooper *et al.*, 1989) but, again, their target (DNA gyrase) is not a conjugation-specific protein, but a general factor in DNA metabolism. These studies demonstrated that it is generally possible to inhibit plasmid transfer, although none of the compounds assayed targeted specifically the conjugative transfer machinery. In general, the lack of a fast, automated screening method that allows large-scale compound testing has hindered the search for conjugation inhibitors. An additional hindrance has been the difficulty in discriminating between compounds that inhibit conjugation by affecting bacterial metabolism and those that exert their action on the conjugation system per se.

In this work we developed an automated high-throughput conjugation assay that discriminates between true conjugation inhibitors and substances that diminish conjugation due to perturbations in cell growth or bacterial physiology. We then used this assay to search for specific conjugation inhibitors within a chemical library of bacterial and fungal extracts. We found that unsaturated fatty acids, a common component of those extracts, are inhibitors of plasmid conjugation. The discovery of efficient conjugation inhibitors can be useful for at least two reasons: (i) they can help us to understand the mechanisms of conjugation and type IV protein secretion and (ii) they may prove useful in controlling the spread of antibiotic resistance.

METHODS

Bacterial strains and plasmids. *Escherichia coli* strains DH5α [F$^-$ *supE44 lacU169* (*ϕ*80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] (Grant *et al.*, 1990) and CSH53 [*ara* Δ(*lac–pro*) *strA thi* (*ϕ*80Δ*lacI*)] were used as donor strains in conjugation experiments. D1210 (F$^-$ *recA hspR hsdM rpsl laqI*^{q}) (Sandler *et al.*, 1980) and CSH53-Rif, a spontaneous rifampicin-resistant derivative of CSH53, were used as recipients. When appropriate, antibiotics were added at the following concentrations: ampicillin sodium salt (100 μg ml^{-1}), kanamycin sulphate (25 μg ml^{-1}), streptomycin sulphate (300 μg ml^{-1}), nalidixic acid (25 μg ml^{-1}). Plasmids used are described in Table 1.

Enzymes and reagents. Restriction endonucleases *Eco*RI, *Pst*I, *Xho*I, *Not*I and *Sal*I were purchased from Roche. Phage T4 DNA ligase was from New England Biolabs. Saturated and unsaturated fatty acids as well as other related compounds were obtained from Sigma-Aldrich.

Construction of pSU2007::Tn*lux*. A DNA segment containing the bioluminescence operon *luxCDABE* from *Photorhabdus luminescens* was excised from plasmid pSB395 (Winson *et al.*, 1998a, b) by *Eco*RI/*Pst*I digestion. The resulting DNA (containing the *lux* operon ribosome-binding site but not its promoter) was cloned into the expression vector pUHE-24, downstream of a strong artificial Lac promoter (P*lac*), yielding plasmid pSM1979 (Deuschle *et al.*, 1986). This promoter is repressible by LacI. Plasmid pSM1979 was digested with *Xho*I/*Pst*I and the fragment containing *lux*, now preceded by P*lac*, was inserted in vector pLow2 cut with *Sal*I/*Pst*I, in order to have the P*lac-lux* construction flanked by *Not*I sites. The resulting plasmid, pSM1981, was cut with *Not*I and inserted into the unique *Not*I site in pUT-miniTn*5* (Gm^r) (de Lorenzo *et al.*, 1990). The pUT construction was mobilized with helper plasmid pRK600 (de Lorenzo & Timmis, 1994) into *Pseudomonas putida* R1, harbouring plasmid pSU2007, a kanamycin-resistant derivative of plasmid R388 (Martínez & de la Cruz, 1988). Resulting transconjugants were subsequently used as donors in a mating experiment with *E. coli*. A mutant derivative of pSU2007 was thus identified where the Tn*lux* insertion into the plasmid does not interfere with conjugative functions. The resulting tagged plasmid (pSU2007-Tn*lux*) exhibited the same conjugation frequency as the parental plasmids pSU2007 and R388.

Conjugation assays. For the plate-mating procedure (Llosa *et al.*, 1991), a 200 μl mixture of equal volumes of donor and recipient cultures, both in stationary phase, was centrifuged and placed onto a GS Millipore filter (0·22 μm pore size) on top of an LB-agar plate for 1 h at 37 °C. Bacteria were washed from the filter and plated on selective media.

For the *lux*-monitored conjugation procedure, donor cells contained plasmid pSU2007::Tn*lux* and pUC18::*lacI*^{q} (so expression of the *lux* operon was completely repressed and donor bacteria were non-luminescent). Upon conjugation, pSU2007::Tn*lux*, but not pUC18::*lacI*^{q}, moves to the recipient cell, resulting in expression of luminescence in transconjugants. Luminescence was measured as arbitrary light units (ALU) using either an image-capturing system (Chemidoc; Bio-Rad) or a microplate luminometer (either Fluoroskan Ascent from Thermolab Systems, or Victor2 from Perkin Elmer).

For high-throughput conjugation assays in the presence or absence of potential inhibitors, 96-well microtitre plates were used. Extracts were dissolved in ethanol/DMSO (75 : 25, v/v) and 10 μl aliquots dispensed into wells prior to addition of 300 μl molten LB agar. Plates were stored at 4 °C overnight prior to use to allow for diffusion of the compounds. To initiate mating, 10 μl of a 1 : 1 (v/v) mixture of donor and recipient bacteria in stationary phase was inoculated on the surface of individual wells. Conjugation was allowed to proceed for 3 h at 37 °C.

RESULTS

Design of an automated conjugation assay

Plasmid R388 was selected for the development of an automated conjugation assay because (i) it contains one of the simplest bacterial conjugation systems at the genetic level and thus the inhibition assay will target the most widely shared proteins in conjugation, (ii) its type IV secretion system machinery is similar to the canonical VirB system of the *Agrobacterium tumefaciens* Ti plasmid (Christie & Vogel, 2000), and (iii) the atomic structures of two key proteins in conjugation (the relaxase, TrwC, and the coupling protein, TrwB) (Gomis-Ruth *et al.*, 2001; Guasch *et al.*, 2003) are known for this system, so possible inhibitors of these targets could be subsequently analysed at the atomic level with protein-inhibitor co-complexes.

The principle of the conjugation detection assay relies on the production of visible light. pSU2007::Tn*lux*, a derivative of

Table 1. Plasmids

Plasmid	Relevant characteristics	Reference
pKM101	Deletion derivative of R46, TRA_N, Ap^r	Winans & Walker (1985)
pLow2	Cloning vector	Hansen *et al.* (1997)
pOX38Km	Derivative of F, TRA(F^+), IncFI, Km^r	Chandler & Galas (1983)
pSB395	pRK415::*luxCDABE*, Tc^r	Winson *et al.* (1998b)
pSM1979	pUHE 24-2::*luxCDABE*, Ap^r Cm^r, pMB1	This work
pSM1981	pLow2::$P_{A1\text{-}04/03}$*luxCDABE*	This work
pSU2007	Derivative of R388, TRA_W, IncW, Tp^r Km^r	Martínez & de la Cruz (1988)
pSU2007::Tn*lux*	pSU2007::*luxCDABE*, TRA_W, IncW, Tp^r Km^r Gm^r	This work
pSU4628	CloDF13::Tn*AΔEco*RV, Rep (CloDF13), Ap^r	Cabezón *et al.* (1997)
pSU5024	pET3a::*eex* Ap^r	This work
pUHE 24-2	Expression vector, Ap^r Cm^r, pMB1	H. Bujard, University of Heidelberg
pUT-miniTn*5*	pUT-mini Tn*5*, Gm^r	de Lorenzo *et al.* (1990)
pUT-miniTn*5L*	pUT-mini Tn*5*::$P_{A1\text{-}04/03}$*luxCDABE*, Gm^r	This work
R6K drd-1	Transfer-derepressed mutant of R6K, TRA_X (*drd*), IncX, Ap^r	Avila *et al.* (1996)
RP4	Wild-type plasmid, TRA_P, IncP, Ap^r Km^r Tc^r	Datta *et al.* (1971)

plasmid R388, contains a *lux* operon under the control of a *lac* promoter. *lux* expression in conjugative donor cells is repressed by the *lac* repressor LacI carried in a co-resident and non-mobilizable multicopy plasmid (pUC18::$lacI^q$). Upon conjugation, pSU2007::Tn*lux*, but not pUC18::$lacI^q$, is transferred to recipient cells; thus light is produced exclusively in transconjugant cells. Donor cells produce relatively low amounts of light (0·1 ALU) when compared to the transconjugant population after a 1 h mating period (18 ALU). This difference implies more than a 2-log enhancement in light production, so inhibition of the process can be easily quantified.

The assay was optimized by analysing a series of variables. First, different *E. coli* strains were tested as donor and recipients. CSH53 showed optimal results (the peak of light was more stable, more reproducible and of longer duration). Other strains (e.g. DH5α) produced more intense peaks, but light production adversely affected cell growth. The kinetics of plasmid transmission was analysed by following light production versus time (Fig. 1). Light emission increased significantly over donor strain levels from about 40 min and reached a maximum at about 500 min, before cells entered stationary phase. The maximal difference in light emission between donor strain and conjugation mixture ranged between 2·5 and 3 logs.

Second, it was confirmed that light production was directly related to the number of light-emitting cells. CSH53 cells containing pSU2007::Tn*lux* were subjected to twofold serial dilutions and mixed with 10^7 non-luminescent CSH53 cells, and light emission was measured. Since R388 does not mediate conjugation in liquid media, these mixes contain decreasing concentrations of luminescent cells but the same total cell density. Light emission was measured and the total number of luminescent cells in each dilution was determined by plating. The results (Fig. 2) reflect a linear relationship between number of transconjugants and light emission. Thus, inhibition of light emission is a quantitative indicator of conjugation inhibition.

Third, effects of solvents used to deliver the inhibiting compounds were tested. The assay was unaffected by DMSO (5 % final concentration), methanol (10 %) or a mixture of both. Aeration had a pronounced effect on light production. Best reproducibility was obtained when a gas-permeable membrane seal was used on lidless microtitre plates.

To obtain proof of principle that light production can be reduced in response to conditions limiting conjugation, we used CSH53 recipient bacteria carrying plasmid pSU5024, which contains the R388 entry-exclusion gene (*eex*) cloned in expression vector pET3A. The entry-exclusion protein is a small polypeptide able to prevent conjugal transfer of R388

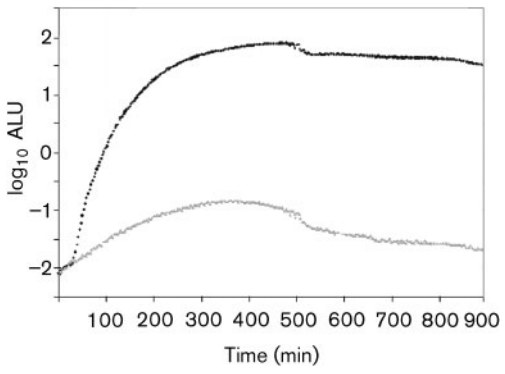

Fig. 1. Lux-monitored conjugation. The experiment was carried out as described in Methods for the Lux-monitored assay. The time-course for light emission in conjugation mixes (black points) and donors alone (grey points) is shown. Luminescence is indicated as the decimal logarithm of the arbitrary light units (ALU) obtained. A maximal difference of 3 logs was obtained after 5 h incubation at 37 °C.

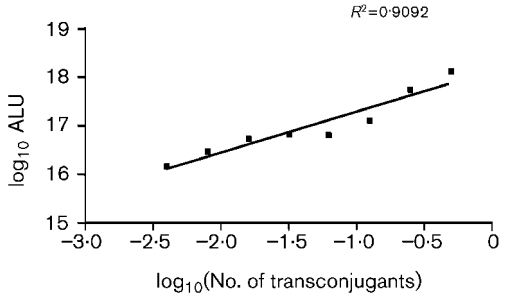

Fig. 2. Relationship between number of transconjugants and light emission. CSH53-Rif (R388::Tn*lux*) cells were grown in LB to OD_{600} 0·6 and subjected to serial dilution in a culture (OD_{600} 0·6) of CSH53-Rif non-luminescent cells. Light production was measured and plotted against the number of luminescent cells present. The result underscores the linear relationship between the number of light-producing cells and light emission as measured from the mixture.

when residing in a recipient cell. No light emission ($<0·1$ ALU) was observed in the conjugation assay when recipient bacteria expressed this protein (data not shown). This experiment demonstrated that the automated conjugation assay can be also used for the screening of genetic conditions affecting conjugation.

Screening a validation set of 224 reference chemicals

As a first application for the automated conjugation assay we tested a set of 224 common chemicals (Table 2). The assay was carried out as described in Methods, with compounds added at 62·5 μg ml^{-1} final concentration. In order to distinguish compounds affecting conjugation from those that affected either cell growth or the process of light production, a control assay was developed, which was run in parallel to the primary assay for each of the tested compounds. In the control assay, CSH53 cells containing pSU2007::Tn*lux* (but not pUC18::*lacI*^{q}) were grown for 2 h in the presence of the potential inhibitor, and light production was measured. Any condition affecting cell growth, plasmid stability, *lux* expression or the light production reaction would be detected as a decrease in light production in the control assay. The control assay was validated by the use of several antibiotics including inhibitors of replication (bleomycin, coumermycin), transcription (rifampicin, actinomycin), protein synthesis (tetracycline, chloramphenicol) and compounds affecting membrane integrity (polymixin, patulin), among others. A compound was considered a conjugation inhibitor if it reduced light production by 95 % or more in the primary assay, but caused a reduction of less than 50 % in the control assay. All measurements were carried out at least in triplicate. When the 224 compounds in the validation plate were screened (Table 2), and compounds positive in the control assay were discarded, only two (oleic and linoleic acids) were found to be true conjugation inhibitors.

Assay of the NatChem extract library

Fig. 3 shows a flowchart for the high-throughput process that was undertaken to search for conjugation inhibitors. The NatChem library includes a large collection of extracts obtained from different bacteria (mainly actinomycetes) and fungi, cultured in different media. 12 000 extracts were assayed by both primary and control assays. A hit rate of 1·4 % was obtained, representing 161 extracts deriving from 139 different organisms. From these, 48 extracts were selected to represent a wide diversity of organisms and extraction conditions. Scale-up fermentations of the appropriate organisms were performed, bulk harvested biomass was extracted in methanol (2 litres) and 30 ml aliquots were fractionated by HPLC. Thus, each of the 48 crude extracts was divided into 24 fractions, giving a total of 1152 fractions. HPLC fractionation separated compounds according to hydrophobicity, so the more hydrophobic the compound the higher the fraction in which it eluted. The 1152 fractions were tested by primary and control assays; 52 active fractions were selected and progressed to secondary assays.

Secondary assays

Active fractions obtained by screening the NatChem library were tested by classical plate mating assays using a set of prototype conjugative plasmids. In addition to R388, plasmids R1 and RP4 were selected as prototypes of IncF and IncP plasmids, respectively. *Streptococcus agalactiae* plasmid pMV158 was transferred to *Enterococcus faecalis* and used as a prototype of a Gram-positive plasmid. However, most of the fractions tested inhibited growth of the *Ent. faecalis* strain used for pMV158 mating, so results with this plasmid were uninformative.

Only one of the 52 selected fractions (*Mollisia ventosa*-Fx14) inhibited conjugal transfer of the three plasmids R388, R1 and RP4. Most fractions were active against R388 and R1, but not against RP4. They were almost always late fractions of the corresponding extracts and in several cases chemical fingerprinting showed them to contain unsaturated fatty acids, principally linoleic acid. Four of these extracts were selected for further work because they contained active early fractions, which could contain different types of compounds. The five prioritized extracts and their active fractions are shown in Table 3. They were refermented and the corresponding extracts fractionated for chemical fingerprinting. The main results are shown also in Table 3. As can be seen, only two compounds were found to be pure in the analysed fractions. Several fractions contained linoleic acid. Fraction *Sistotrema semanderi*-Fx21 contains an uncommon unsaturated fatty acid, and was interesting because it could represent a novel structure. The rest of the fractions (including *Mollisia ventosa*-Fx14) were complex in composition, and were not analysed further.

Fraction *S. semanderi*-Fx21 was selected for further studies because of its purity. The chemical fingerprint showed one main peak, indicating that there was one major compound.

Table 2. Screening results of the conjugation and control assays on a reference set of chemicals

A + score indicates a light emission value under the 95 % cut-off in the primary assay (P) or 50 % cut-off in the control assay (C). A compound was considered a conjugation inhibitor if it decreased light emission under the threshold in the primary assay but not in the control assay, or a non-specific inhibitor if it decreased light emission in both assays. All assays were carried out in triplicate.

Compound	P	C	Compound	P	C	Compound	P	C
Teicoplanin			Muscarine chloride			Me-OH-xanthone-carboxylate		
Fosmidomycin			Muscimol			3-Ethyl-6-hydroxylphthalide		
Dihydrostreptomycin			Fumarprotocetraric acid			N5014 metabolite drimane		
Vancomycin			Usnic acid			BE18257 A		
Tetracycline	+	+	Secalonic acid			Fatty acid ester phomalactone		
Kanamycin			Physcion			N5444 metabolite terpenoid		
Streptomycin		+	Ferroxianmine (desferri-)		+	Zeaeralanone		
Bacitracin		+	Val-Asp [*N*-(1-desoxyfructosyl)]			Zeaeralenol		
Cerulenin		+	Gramicidin D			Striatal B		
Penicillamine			Bestatin			Uridine		
Antimycin A		+	Cyclosporin A			Striatal A		
Griseofulvin			Thielavin B			Cyclopiazonic acid		
Rifampicin		+	Vinblastine			Streptopyrrole derivative	+	+
Aphidicolin			Ergotamine tartrate			Furanone derivative		
Chalcomicin			Minocycline	+	+	Enniatin B		
Actinomycin D		+	Paxilinine			Verruculogen		
Amphotericin B			Tetrahydroauroglaucine			XR379 fatty acid side chain		
Nystatin			Dihydroauroglaucine			9-Methoxystrobilurin E		
Gramicidin S		+	Apigenin			Phomalactone		+
Kasugamicin			Quercetin			Radicinol/radicin		+
Anisomycin			Linoleic acid	+		Cycloaspeptide A		
Novobiocin			Oleic acid	+		Pimprinine		
Rifamycin SV		+	Haematoporphyrin IX zinc			Spirodihydrobenzofuran		
Oleandomycin		+	Oxalic acid			Astichlorin B		
Chloramphenicol	+	+	Sterol sulphate		+	Trichostatin		
Neomycin sulphate		+	Fumagiline DCM			Piericidin A1		
Oxytetracycline	+	+	Sclerotiorin		+	Myxothiazol		
Erythromycin			Curvularin		+	Cytochalasin E		
Mitomycin C			Linolenic acid			Methoxy-XR587		
Penicillin G			Genistein			Monascorubramine		
Cycloserine (D)		+	Ergosterol		+	Daidzein		
Polymyxin B sulphate	+	+	Arternariol monomethyl ether		+	Aspochalasin E		
Azaserine			Brefeldin A			Aspochalasin C		
Puromycin			Zeaenol			Hursutanes		
Lincomycin hydrochloride		+	Oxozeaenol		+	Hursutanes		
Calcium ionophore A23187		+	Xenovulene B			Emericin III		
Nigericin			5Z 11E7-oxo-zeaenol		+	Emericin IV		
Cycloheximide		+	TPI-1 and TPI-2	+	+	Lasalocid Na salt		
Stroptozotocin			Verrrucarin H			Rubratoxin		
Gentamicin sulphate			Monensin			Aurovertin		
Tobramycin	+	+	Chromomycin A3			Moniliformin		
Ristocetin			Doxorubicin			Tenuazonic acid		
Spiramycin		+	Hygromycin B	+	+	Wortmanin		
Nonactin			Salinomycin			Penitrem A		
Zearalenone			Nikkomycin Z			Thiostrepton		+
Cephalosporin C		+	Castanospermine			Ochratoxin		
Mycophenolic acid			Cordycepin			Picrotoxin		
Fusidic acid			Clindamycin hydrochloride			Streptonigrin	+	+
Camptothecin			Forskolin			Echinomycoin		
Phosphomycin		+	Chlortetracycline	+	+	Spectinomycin	+	+

Table 2. cont.

Compound	P	C	Compound	P	C	Compound	P	C
Fusaric acid		+	Eblelactone A			Harveynone metabolite		
Actinonin			Xanthone derivative		+	Quinomycin A		
Gliotoxin	+	+	Xenovulene C			Depsipeptide		
Oligomycin			Xenovulene A			8-Acetoxyroridin H		
Tunicamycin			Terrein		+	7,7′-Methoxyrugulosin	+	+
Citrinin			Chaetoglobosin A			β-Rubromycin		
Aflatoxin B1			Elaiophylin			Erythronolide B		
Patulin		+	Phomalactone derivative	+	+	Bleomycin A2 hydrochloride	+	+
Valinomycin			Cyclo-L-prolyl-L-tryptophyl			Blasticidin S hydrochloride		
Tubecidin			Kirromycin			Lasalocid Na salt		
Cytochalasin B			Emodin		+	Paromycin sulphate		
Trichothecin			Mycolutein			Spectinomycin 2HCl	+	+
Helvolic acid			Luteoreticulin			Tylosin tartrate		+
Roridin A			Geldanamycin			Narasin		
Filipin			L-671,776			Amikacin sulphate		+
Daunomycin hydrochloride		+	Asterric acid			XR733 analogue		
Leupeptin hemisulphate			Monorden			XR733		
Pepstatin A			Verrucarin A			Manumycin A		
Alamehicin			Sterigmatocystin			Coumermycin A1	+	+
Capropmycin sulphate			Sydowinin B			Myriocin		
Mevastatin			Alternariol			Nogalamycin		
Ophiobolin A			Sclerotionin derivative					
Lithium clavulanate			5-Chloroisorotiorin					
Mevinolin			N36923 metabolite Cubist isolate					
1-Deoxynojirimycin hydrochloride			Spirostaphylotrichin A					

We were able to resolve its structure by nuclear magnetic resonance (for details, see the supplementary data with the online version of this paper). The compound was identified as dehydrocrepenynic acid (DHCA), a C_{18} fatty acid with double bonds at positions 9 and 14, and a triple bond at carbon 12 (Fig. 4). It was first described by Bu'Lock & Gregory (1959) as a natural acetylenic acid. The inhibition spectrum of the purified compound was the same as that of the full fraction and no effect was detected on the control assay, indicating that DHCA is a bona fide conjugation inhibitor.

An analysis of the inhibitory effect of fatty acids on bacterial conjugation

Most active fractions found by screening the NatChem collection contained unsaturated fatty acids, mainly linoleic acid. In the analysis of the library, the identification of DHCA emphasized the importance of this kind of compound, so we decided to further characterize their conjugal inhibitory activities. To evaluate the functional importance of the different chemical groups, we analysed the inhibitory effect of oleic acid, linoleic acid and related compounds on R388-mediated conjugation. First we tested some compounds with hydrophobic linear carbon chains, such as hexanol, hexane, hexadecane and squalene, to prove that inhibition was not due to unspecific effects of long hydrocarbon chains on the bacterial membrane. They all tested negative in conventional plate mating assays. Saturated fatty acids, such as caproic acid (C12 : 0), lauric acid (C16 : 0) and palmitic acid (C17 : 0), also failed to exert an inhibitory effect on R388-mediated conjugation when using either the *lux*-monitored assay or the conventional plate mating assay [we were unable to test stearic acid (C18 : 0) since its melting temperature is around 68 °C]. Of the unsaturated fatty acids tested, the monounsaturated oleic acid (C18 : 1Δ9) and diunsaturated linoleic acid (C18 : 2Δ9,12) both inhibited conjugal transfer of R388. Interestingly, vaccenic acid (C18 : 1Δ11), differing from oleic acid only in the position of its double bond, did not produce an inhibitory effect. These results suggest that the double bond at position 9 may be essential for inhibitory activity, with polyunsaturated fatty acids being more potent than monounsaturates.

The MICs of the unsaturated fatty acids were also determined. Oleic and linoleic acids had MIC_{98} values of about 400 μM, while the MIC of DHCA was 70 μM (data not shown). At a final concentration of $2 \times MIC_{98}$, R388 transfer frequency decreased by a factor of 20 with oleic acid, while 200-fold and 350-fold reductions were observed for the polyunsaturated fatty acids linoleic acid and DHCA respectively.

Finally we tested the spectrum of plasmids inhibited by linoleic acid and DHCA (Table 4). As expected, the

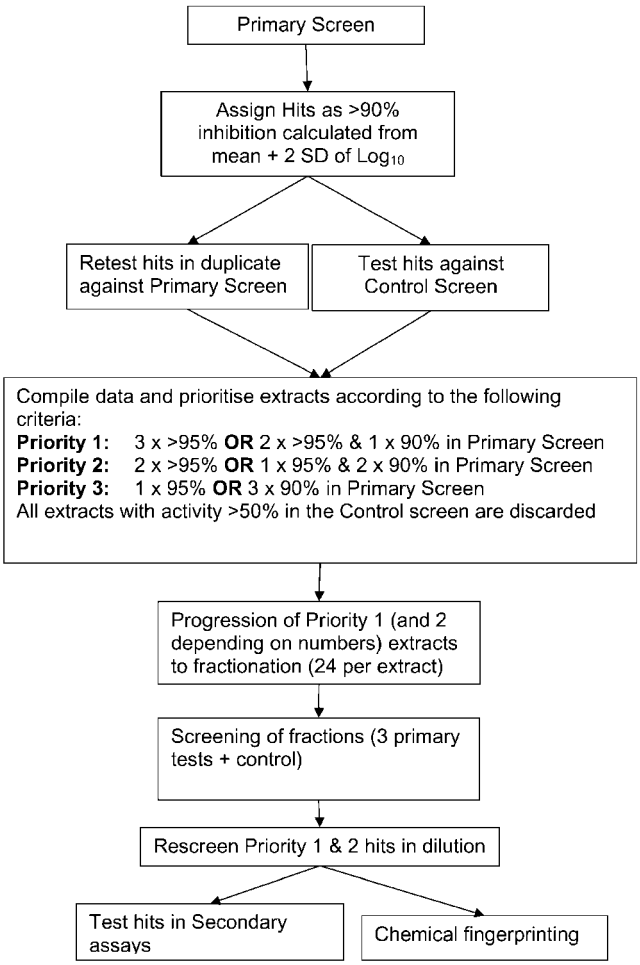

Fig. 3. Flowchart for high-throughput-screening of the NatChem library.

compounds strongly inhibited conjugation mediated by R388 and the F-plasmid derivative pOX38. The effect of DHCA was always at least twice as pronounced as that of linoleic acid. As expected also, there was no inhibition of plasmid RP4 transfer. Consistent with that observation, plasmid R6K (most similar to RP4) was not inhibited either. All these results are consistent with the notion that unsaturated fatty acids may be targeting the plasmid Dtr systems, which are in common between R388 and F, but distantly related to those of RP4 and R6K (Francia *et al.*, 2004). This notion was reinforced by the observation that mobilization of plasmid CloDF13, which requires the R388 Mpf system for its transmission, was not inhibited when a donor strain contained both plasmids. In this situation, R388 was inhibited while CloDF13 was not (Table 4). The Dtr components as a site of action for these substances became less likely, however, when their effect on conjugative transfer of plasmid pKM101 (whose Dtr is more similar to R388 and F than to RP4) was compared and no inhibition was observed (Table 4).

DISCUSSION

Conjugation is a main player in the horizontal transfer of multiple antibiotic resistance determinants among pathogenic bacteria. Compounds able to interfere with this process may potentially prove useful in controlling the spread of antibiotic resistance. Nevertheless, no significant progress has been made up to now in the search for conjugation inhibitors, due at least in part to the lack of a conjugation assay amenable to large-scale screening of compound libraries. Thus, we sought to develop an automated assay for bacterial conjugation and to apply it for high-throughput screening of a bacterial and fungal extract library in a search for bacterial conjugation inhibitors.

The *lux*-based conjugation assay we developed is well-suited for this purpose. Light production can be measured continuously in real time. Conjugation frequencies can be estimated directly and the assay is sensitive and easily automated. We used it to screen the NatChem compound library, a collection of 12 000 fungal and bacterial extracts. During assay validation it was discovered that linoleic and oleic acids (*cis*-unsaturated C_{18} fatty acids) were true inhibitors of conjugation mediated by plasmid R388. These compounds were also present in many extracts of the NatChem library, as fungi often produce them in high amounts. This was unfortunate, because many NatChem hits were produced by unsaturated fatty acids, largely precluding the finding of additional classes of compounds.

Nevertheless, NatChem library screening allowed us to discover an atypical fatty acid that was a bona fide inhibitor of R388-mediated conjugation. Its structure, solved by NMR, revealed dehydrocrepenynic acid. DHCA is also a C_{18}, *cis*-unsaturated (9,12,14) fatty acid, with the peculiarity of having a triple bond between C-12 and C-13. The inhibitory potential and spectrum of DHCA were essentially the same as those of oleic and linoleic acids. Saturated short- and long-chain fatty acids, or other organic compounds related to them, showed no significant inhibitory effect, so we believe that a carboxylic group, chain length and position of the double bonds are essential features of this class of conjugation inhibitors.

Long-chain fatty acids are actively transported into the *E. coli* cytoplasm by a highly specific system (Black & DiRusso, 2003). FadL is an outer-membrane protein that binds long-chain fatty acids with high specificity and facilitates their transfer across the membrane. Translocated fatty acids cross the periplasmic space and the inner membrane via an unknown mechanism, although there is some evidence of a H^+/fatty acid cotransporter (fatty acid transport depends on membrane potential). Most transported fatty acids are activated by FadD, an inner-membrane-associated acyl-CoA synthetase, and directed to β-oxidation. A small proportion of the exogenous fatty acids are incorporated directly into the phospholipid production system by the acyl-acyl carrier protein synthetase. This implies that most of the oleic,

Table 3. Prioritized fractions obtained after screening the NatChem library

Five crude extracts found to be active in the primary assay, but with no significant effect in the control assay, were subjected to chemical fractionation and tested for their effects on the conjugal transfer of three reference plasmids. The table indicates the generic name of the source organism (when known), the phase of the fermentation from which the extract was obtained, the activity against R388, RP4 and R1 conjugation (Y, active; N, inactive), and the main characteristics of the fraction as inferred from chemical fingerprinting.

Generic name	Fermentation	Active fractions	R388	RP4	R1	Fingerprint
Sistotrema semanderi	Solid	Fx21	Y	N	Y	High purity, one main peak
Mollisia ventosa	Solid	Fx14	Y	Y	Y	Complex fraction (5 or more components)
Pyrenopeziza sp.	Solid	Fx23	Y	N	Y	High purity, linoleic acid
		Fx10	Y	N	N	Complex fraction
Unknown	Liquid	Fx22	Y	N	Y	High purity, linoleic acid
		Fx15	Y	N	Y	One main peak + fatty acids
Unknown	Liquid	Fx15	Y	N	Y	High purity, linoleic acid
		Fx9	Y	N	N	Low purity, no main peak
		Fx16	Y	N	N	Low purity, no main peak
		Fx21	Y	N	Y	Three components likely to be fatty acids
		Fx22	Y	N	Y	Low purity, no main peak
		Fx23	Y	N	Y	High purity, linoleic acid

Table 4. Effect of linoleic acid and DHCA on plasmid conjugation

Derivatives of CSH53 containing the plasmids indicated were conjugated to CSH53-Rif for 1 h, using the plate-mating procedure. Conjugations were carried out on LB agar, LB agar supplemented with linoleic acid at a final concentration of 1 mM and LB agar supplemented with DHCA at a final concentration of 1 mM. Conjugation frequencies obtained represent the mean of three different experiments.

Plasmid	Conjugation frequency		
	LB	LB + linoleic acid (1mM)	LB + DHCA (1 mM)
pSU2007	$7{\cdot}0 \times 10^{-1}$	$3{\cdot}0 \times 10^{-3}$	$2{\cdot}0 \times 10^{-3}$
pKM101	$6{\cdot}0 \times 10^{-1}$	$9{\cdot}0 \times 10^{-1}$	$1{\cdot}1 \times 10^{-1}$
R6K drd	$6{\cdot}0 \times 10^{-1}$	$4{\cdot}0 \times 10^{-2}$	$1{\cdot}0 \times 10^{-1}$
RP4	$2{\cdot}3 \times 10^{-2}$	$9{\cdot}3 \times 10^{-3}$	$1{\cdot}4 \times 10^{-1}$
R388 + CloDF13*	$1{\cdot}6 \times 10^{-2}$	$7{\cdot}5 \times 10^{-3}$	$3{\cdot}0 \times 10^{-2}$
pOX38	$7{\cdot}0 \times 10^{-1}$	$6{\cdot}6 \times 10^{-3}$	$1{\cdot}4 \times 10^{-3}$

*The figures in this row refer to CloDF13 mobilization. The R388 plasmid transferred exactly as pSU2007 above.

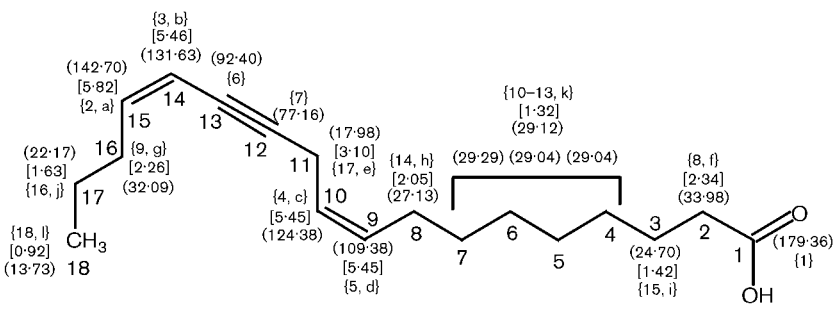

Fig. 4. Chemical structure of DHCA as obtained by NMR. ^{1}H and ^{13}C NMR assignments are shown. Parentheses denote a ^{13}C chemical shift relative to solvent at 7·26 p.p.m., while square brackets indicate a ^{1}H chemical shift relative to solvent at 77·02 p.p.m. Numbering of the carbons in the chain is also shown. A detailed account of the NMR results that led to the structure shown is provided as supplementary data with the online version of this paper.

linoleic and DHCA acids supplied as inhibitors will be degraded when they enter the bacterial cell. A small amount, however, will reach the phospholipid pool and perhaps cause a change in lipid composition of the membranes. Although we have no evidence for the mechanism of action of these compounds, some hypotheses can be suggested. A culture medium highly enriched in fatty acids may cause a perturbation in the general physiology of *E. coli* (in osmotic pressure control, membrane potential or energetic balance) that impairs conjugation as a pleiotropic effect. However, and perhaps significantly, only certain conjugative plasmids are affected by these compounds (i.e. F and R388) while others are not inhibited (RP4). Thus, a general metabolic disturbance as the cause of inhibition is unlikely.

Alternatively, unsaturated fatty acids may directly target the conjugation machinery. It is known that unsaturated fatty acids can affect the function of some proteins associated with the bacterial membrane. For instance, DnaA, an ATPase involved in chromosome replication initiation in *E. coli*, specifically binds phospholipids containing oleic acid. This interaction is essential to stimulate DnaA ADP/ATP exchange, and inhibitors of unsaturated fatty acid synthesis produce a sharp decrease in DnaA activity that can be restored by providing exogenous oleic acid (Yung & Kornberg, 1988). Since bacterial conjugation requires the active participation of at least three membrane ATPases (TrwB, TrwD and TrwK in the case of R388), it is possible that phospholipids containing unsaturated fatty acids have a more direct role in the mechanism of conjugation by specifically interacting with these proteins. In fact, preliminary biochemical data from our laboratory indicate that the traffic ATPase TrwD is inhibited by unsaturated fatty acids in a non-competitive manner (C. Machón and others, unpublished).

Our results have shown that unsaturated fatty acids inhibit conjugation mediated by plasmids F and R388, while conjugation mediated by plasmids pKM101, RK6 and RP4 is insensitive to these compounds. The absence of an effect of linoleic acid and DHCA on R388-mediated mobilization of plasmid CloDF13 suggests that the main target of these compounds is the DNA transfer replication (Dtr) machinery. Plasmid CloDF13, like most mobilizable plasmids, uses its own Dtr machinery but takes advantage of the R388-built transport channel (the so-called Mpf system) to achieve its own transfer. The fact that it is not inhibited by DHCA while R388 transfer (from the same donor cell) is inhibited, suggests that the target is more likely to be a component of the Dtr system than the Mpf system. The closer relationship between the Dtr modules of R388 and F as compared to RP4 supports this possibility. However, plasmid pKM101 also shows a close degree of homology to the R388 and F Dtr systems, while its conjugal transfer was not affected by unsaturated fatty acids. Although we propose this obviously simplistic working hypothesis, we are aware that the mechanism underlying the inhibition might be more subtle or complex. At present, the mode of action of the inhibitors is simply unknown. Our current research aims to identify the molecular targets of unsaturated fatty acids in R388-mediated conjugation and characterize their interactions in the hope of a rational design of better inhibitors.

The availability of a robust and sensitive high-throughput conjugation assay will allow massive screening of compound libraries in new searches for compounds affecting bacterial conjugation and type IV protein secretion. The assay can also be used for analysis of libraries of bacterial mutants in a search for genes affecting conjugation (or type IV secretion) and its regulation using a variety of plasmids or host strains. All of this will result in better knowledge of the mechanisms and physiological control of bacterial conjugation and, perhaps, in the discovery of compounds that may help us to control the spread of antibiotic resistance.

ACKNOWLEDGEMENTS

R. F. L. was supported by an FPU fellowship from the Spanish Ministry of Education. C. M. was supported by a fellowship from the Basque Government (Spain). This work was supported by grant BMC2002-00379 from the MEC (Spain) to F. C. All partner laboratories were supported by a European Union grant from the Fifth Framework Programme (QLK2-CT-2000-01624).

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