10-Undecanhydroxamic acid, a hydroxamate derivative of the undecanoic acid, has strong antimicrobial activity through a mechanism that limits iron availability

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Abstract
Undecanoic acid (UDA) is a fatty acid with significant antimycotic activity. In this work we have synthesized 10-undecanhydroxamic acid, a hydroxamate derivative of the UDA, and tested its antimicrobial activity on different microorganisms. Our results demonstrate that this compound has higher efficacy than UDA against a variety of fungi and bacteria. Analysis of the intracellular concentration of protein involved in iron transport in Salmonella enterica serovar Typhimurium suggests that its antimicrobial effect actually relies on the ability to chelate iron ions, providing an efficient mechanism to interfere with microbial growth.

Introduction
Iron is an essential element for the growth of all living organisms as it plays crucial roles in many cellular functions, including the transport and storage of oxygen, electron transport processes and a variety of catalytic processes (Schaible & Kaufmann, 2004). Under physiological conditions, i.e. at neutral pH and in the presence of oxygen, free extracellular iron is rapidly oxidized from Fe^{2+} to Fe^{3+} and forms insoluble hydroxides that limit iron availability for microorganisms. Microorganisms have evolved various mechanisms to counteract the problems imposed by their iron dependence, allowing them to achieve adequate iron supply under a wide range of iron regimes (Fischbach et al., 2006). Highly efficient iron acquisition systems are used to obtain iron from the environment under iron-restricted conditions. In many cases, these strategies involve the secretion and internalization of extracellular ferric chelators called siderophores (Faraldo-Gomez & Sansom, 2003). The synthesis and secretion of such molecules, as well as the expression of a large set of genes controlling intracellular iron homeostasis, is mainly controlled by the global iron homeostasis transcriptional regulator Fur (Escolar et al., 1999), whose ability to interact with specific DNA sequences is regulated by the binding of Fe^{3+}. As free iron is extremely limited in the mammalian host, the problem of an efficient acquisition of this metal is particularly acute for intracellular pathogens (Wooldridge & Williams, 1993). It has been estimated that about 7% of the Salmonella genes, including several virulence factors, are directly or indirectly regulated by iron and most of them are controlled by Fur (Bjarnason et al., 2003).

The relevance of adequate iron recruitment for microbial survival and multiplication and the fact that natural host defences limit the growth of infectious agents through strategies based on metal sequestration, suggest that microorganisms may be vulnerable to interventions that disrupt iron acquisition. Severe iron limitation, in fact, may cause a
reduction in the activity of a large number of essential proteins (including cytochromes, tricarboxylic acid cycle metalloenzymes and antioxidant enzymes such as catalases and superoxide dismutases) that require this metal ion as cofactor. On this basis, several attempts have been carried out to develop new antimicrobial strategy based on iron chelation (Sigle et al., 2005; Meyer, 2006). In this work we have explored the possibility to increase the efficacy and spectrum of activity of an antimicrobial fatty acid by the introduction of an iron-chelating chemical group potentially able to interfere with iron homeostasis. The effectiveness of fatty acids and their derivatives as antimicrobial agents against bacteria and fungi has been known for a long time (Kabara et al., 1972; Garg & Müller, 1993), and a large number of studies have established that long-chain unsaturated fatty acids play a role in natural host defence against pathogens in human milk, skin and mucosal secretions (Thormar & Hilmarsson, 2007). Even though the mechanisms by which fatty acids inhibit microbial growth are poorly understood, these compounds are already employed in antifungal formulations for the treatment of mycotic infections (i.e. keratomycoses) and current investigations are actively evaluating the effects of fatty acids on different microorganisms (Lee do et al., 2007; Yang et al., 2007; Nobmanna et al., 2009).

Undecanoic acid (UDA) is one of the most effective fatty acid compounds. It has been suggested that its antimycotic properties are linked to the ability to inhibit the production of exocellular keratinase, lipase and several phospholipids (Das & Banerjee, 1982). To verify the hypothesis that the insertion of an iron-chelating moiety could enhance the antimicrobial effectiveness of fatty acids, we modified UDA by introducing a hydroxamic group in the molecule. Our results indicate that this hydroxamate derivative of the UDA [10-undecanhydroxamic acid (UHA)] has a higher inhibitory effect on microbial growth than UDA. Moreover we have shown that UHA modulates the expression of some Fur-regulated genes in Salmonella enterica serovar Typhimurium (hereafter referred to as S. Typhimurium), supporting the hypothesis that its strong antimicrobial activity relies on its ability to interfere with iron homeostasis.

**Materials and methods**

**Strains, media, growth conditions and reagents**

All the strains used in this work are listed in Table 1. *Salmonella* and *Escherichia coli* strains were cultured in Luria–Bertani (LB) broth (10 g Bacto tryptone, 5 g yeast extract, 10 g NaCl L$^{-1}$) at 37°C in a Lab-Line Orbit Environ-Shaker and growth was monitored by measuring the OD$_{600}$ nm with a Perkin Elmer Lambda 9 spectrophotometer. For Western blot studies, *Salmonella* was grown in M9 minimal medium (Sambrook & Russell, 2001) supplemented with 0.1% casaminoacid and 0.5% glycerol. *Enterococcus faecalis* strain OG1RF was cultured in brain heart infusion (BHI) medium (Becton Dickinson) at 37°C with aeration. The yeast *Candida albicans* and the fungi *Aspergillus niger* and *Chaetomium globosum* were grown in YPD medium

<table>
<thead>
<tr>
<th>Table 1. Strains used in this study</th>
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<tbody>
<tr>
<td><strong>Salmonella enterica serovar Typhimurium ATCC14028</strong></td>
</tr>
<tr>
<td>MA6926</td>
</tr>
<tr>
<td>SA140</td>
</tr>
<tr>
<td>SA 213</td>
</tr>
<tr>
<td>SA 214</td>
</tr>
<tr>
<td>SA 218</td>
</tr>
<tr>
<td>SA 147</td>
</tr>
<tr>
<td><strong>Escherichia coli K12</strong></td>
</tr>
<tr>
<td>71/18</td>
</tr>
<tr>
<td><strong>Enterococcus faecalis</strong></td>
</tr>
<tr>
<td>OG1RF</td>
</tr>
<tr>
<td><strong>Candida albicans</strong></td>
</tr>
<tr>
<td><strong>Aspergillus niger</strong></td>
</tr>
<tr>
<td><strong>Chaetomium globosum</strong></td>
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</tbody>
</table>

*Where not specified, the source of the strain is this work.*

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Epitope tagging of chromosomal genes

The chromosomal genes of *S. Typhimurium* ATCC14028 *iroB*, *sitA*, *fluf* and *pepT* were tagged at the 3’-terminus by adding a 3XFLAG epitope according to the previously described procedure (Uzzau et al., 2001). The oligonucleotides and plasmids used for epitope tagging are listed in Table 2. Briefly, a fragment containing the 3XFLAG epitope and the kanamycin resistance cassette was amplified from plasmid pSUB11 with the following pairs of oligonucleotides: oli-137/oli-138, oli-140/oli-141, oli-143/oli-144 and oli-125/oli-126. The fragments were purified on Microcon columns and electroporated in *S. Typhimurium* ATCC14028 strain MA6926 pKD46. Recombinants were selected on kanamycin and the correct insertion of the 3XFLAG allele was moved in a clean background by generalized transduction using phage P22 HT/mt205 (Maloy et al., 1996). The *ibvI::Tn10dTac-cat::3XFLAG* allele was transduced in each epitope-tagged strain to have an internal 3XFLAG-kanR cassette, and growth was monitored by measuring the OD600 nm with a Perkin Elmer Lambda 9 spectrophotometer. 2,2’-Bipyridyl was purchased from BDH and UDA from Sigma-Aldrich.

**Western blot and immunodetection of the FLAG epitope**

Preparation of bacterial lysates for Western blot and immunodetection with anti-FLAG monoclonal antibody (Sigma) were carried out as described (Uzzau et al., 2001).

**UHA synthesis**

UHA was prepared by adding a solution of 1.8 g (26 mmol) of hydroxylamine hydrochloride in 5 mL tetrahydrofuran and 3 mL water, to 6.0 mL of 25% NaOH solution (2.0 g, 52 mmol) at 10 °C. The first NaOH equivalent has been added directly to the solution, and the second one has been added dropwise along with 5 mL of 10-undecenoyl chloride (4.72 g, 23 mmol). The temperature was slowly increased to room temperature and the solution was stirred overnight. The floating solid formed was dissolved with 20 mL CHCl3. The organic phase was separated, washed with 50 mL of 1% aqueous acetic acid and dried over anhydrous Na2SO4. The solvent was finally removed under vacuum to afford 3.9 g of UHA (yield 85%). The identity and the purity of the obtained compound were verified by 1H-nuclear magnetic resonance analysis (see Supporting Information, Fig. S1).

### Table 2. Oligonucleotides and plasmids used for *Salmonella* epitope-tagged allele construction

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Sequences or references</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oligonucleotides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>oli-137</td>
<td><em>iroB</em> 3’-end, forward</td>
<td>CGAGGTGGCATAAAGAAGACCATGGACGGGCTGGCGTCATCGGATCAAAAGACCATGACGG</td>
</tr>
<tr>
<td>oli-138</td>
<td>Downstream <em>iroB</em>, reverse</td>
<td>GTGAAGATGAGATATGCGGACACCGCTGGGGTCAGCGCCATATGATATCTCCTTAG</td>
</tr>
<tr>
<td>oli-139</td>
<td>Upstream <em>iroB</em>, forward</td>
<td>CAAGAGATCTGGCGTGGATA</td>
</tr>
<tr>
<td>oli-140</td>
<td><em>sitA</em> 3’-end, forward</td>
<td>CATCGCAACCGGTCACTGCCGAGAGTTCAACACAGGACACCATGACGG</td>
</tr>
<tr>
<td>oli-141</td>
<td>Downstream <em>sitA</em>, reverse</td>
<td>CCCGGTACCTGAAAAATGGCGCCGGCTGCGTGGGTGATGCGCGTATATGATATCTCCTTAG</td>
</tr>
<tr>
<td>oli-142</td>
<td>Upstream <em>sitA</em>, forward</td>
<td>GGTTGAGATCGTCCGCTGATA</td>
</tr>
<tr>
<td>oli-143</td>
<td><em>thuf</em> 3’-end, forward</td>
<td>CCTACCTGCTACGACTCGACCCGGCTGAGTTCAACACAGGACACCATGACGG</td>
</tr>
<tr>
<td>oli-144</td>
<td>Downstream <em>thuf</em>, reverse</td>
<td>CAAAAAGGGAAAGCCTGATAATTGGGCACTGGAGAGGCGCCGCGCATATGATATCTCCTTAG</td>
</tr>
<tr>
<td>oli-145</td>
<td>Upstream <em>thuf</em>, forward</td>
<td>TGGTATACGTCGGCCTGATGAC</td>
</tr>
<tr>
<td>oli-125</td>
<td><em>pept</em> 3’-end, forward</td>
<td>GATGGGTGGTGGCCTGACGCTGACGCTGACGCTGACCTGAGTTCAACACAGGACACCATGACGG</td>
</tr>
<tr>
<td>oli-126</td>
<td>Downstream <em>pept</em>, reverse</td>
<td>CATGCTGACCTGTTGGCTGGGGCCGACCAGAAGGCGCATATGATATCTCCTTAG</td>
</tr>
<tr>
<td>oli-127</td>
<td>Upstream <em>pept</em>, forward</td>
<td>GTTTGACCAAAGTGGAGG</td>
</tr>
<tr>
<td>K1</td>
<td>Internal 3XFLAG-kanR cassette, reverse</td>
<td>Matzanke et al. (2004)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pKD46</td>
<td>λ-red recombinase functions</td>
<td>Datsenko &amp; Wanner (2000)</td>
</tr>
<tr>
<td>pSUB11</td>
<td>3XFLAG-kanR cassette template</td>
<td>Uzzau et al. (2001)</td>
</tr>
</tbody>
</table>
Results and discussion

The effect of UHA on microbial growth was tested on various microorganisms, such as the gram-negative bacteria *E. coli* and *S. Typhimurium*, the gram-positive bacterium *E. faecalis*, the yeast *C. albicans* and the fungi *A. niger* and *C. globosum* (listed in Table 1). The growth was monitored for 24 h for each species either in the absence or in the presence of increasing amounts of UHA by measuring the OD600 nm. The growth curves are presented in Fig. 1. Addition of UHA to the media causes a dose-dependent growth inhibition in all cases, susceptibility being dependent on the microorganism tested. The yeast *C. albicans* displays the highest susceptibility to UHA, as its growth is markedly inhibited already at 0.08 mM (Fig. 1c). Exposure to UHA of the gram-negative bacteria *E. coli* and *S. Typhimurium* (Fig. 1a and b) causes inhibition of growth at 0.2 and 0.4 mM, respectively. Compared with the other tested microorganisms, *E. faecalis* shows the highest resistance to UHA (Fig. 1d), as its growth is strongly inhibited only at a concentration of 1.5 mM. To test the antimicrobial effect of UHA on the ascomycetes *A. niger* and *C. globosum*, which cause disease in a variety of plants and occasionally in humans (Guardo et al., 1995; De Lucca, 2007), fungi were inoculated in YPD medium containing 0.05, 0.5 or 1 mM UHA or ethanol and their growth was monitored for 4 days at room temperature. Growth was completely inhibited at 0.5 mM UHA concentration (data not shown). These results indicate that UHA has an efficient dose-dependent antimicrobial activity over a wide number of microorganisms.

To compare the antimicrobial activities of UHA and UDA we have determined the minimal inhibitory concentration values using the broth dilution method. Overnight precultures of *S. Typhimurium*, *E. coli*, *E. faecalis* and *C. albicans* were diluted in fresh medium (LB, BHI or YPD) containing increasing amounts of UHA or UDA. We have compared the relative efficacy of the two compounds in a concentration range between 0.1 and 2 mM. As shown in Table 3, UHA is more effective than UDA on all the microorganisms tested and shows a broad spectrum of activity.

The presence of a hydroxamic group on UHA suggests that its efficient antimicrobial effect compared with that of UDA relies on its ability to interfere with iron homeostasis. To test this hypothesis, we analysed the ability of sublethal doses of UHA to modulate the expression of a set of well-characterized iron-dependent genes in *S. Typhimurium*, including *iroB*, *sitA*, *fluF* and *pepT*. The *iroB* gene, a component of *iroA* gene cluster involved in the modification and transport of enterobactin, encodes for a glycosyltransferase induced under iron-limiting conditions and repressed
by Fur (Fischbach et al., 2005). The sitA gene, which encodes the periplasmic binding component of a transport system with high affinity for Fe$^{2+}$ (Janakiraman & Slauch, 2000), and fhuF, which encodes a cytoplasmic FeS cluster protein involved in the mobilization of ferric iron from hydroxamate siderophores (Matzanke et al., 2004), are both repressed under iron-rich growth conditions in a Fur-dependent manner. In contrast, the aminopeptidase PepT is positively regulated by Fur (Bjarnason et al., 2003).

The expression of these genes was indirectly analysed by measuring the intracellular accumulation of the epitope-tagged proteins in Salmonella strains modified by introducing the sequence encoding for the 3XFLAG epitope at the 3’-end of each chromosomal gene, as described in Materials and methods. The epitope-tagged strains were grown for 6 h in LB medium and then diluted 1:500 in fresh LB medium (iron-rich condition), in LB supplemented with the iron chelator 2,2'-bipyridyl (iron-limiting condition), in iron-supplemented LB medium (FeCl$_3$ 0.1mM) or in LB with increasing amounts of UHA (from 0.1 to 0.4 mM). After 18 h of growth, aliquots corresponding to $2.5 \times 10^8$ bacteria were collected, lysed in Laemmli buffer and treated for sodium dodecyl sulphate polyacrylamide gel electrophoresis and Western blot. The accumulation of each protein was evaluated by immunostaining with anti-FLAG antibodies as described (Uzzau et al., 2001), in comparison with the accumulation of the Cat-3XFLAG protein, which is insensitive to the addition of either 2,2'-bipyridyl, FeCl$_3$ or UHA (Fig. 2). When Salmonella is grown in LB, IroB is produced at a low level (Fig. 2a, lane 1) and is almost repressed in iron-supplemented LB (lane 3), but its intracellular amount increases in cells exposed to UHA (Fig. 2a, lanes 4–6) or to 2,2'-bipyridyl (Fig. 2a, lane 2). Albeit to a minor extent, the same behaviour is observed for FhuF. In fact, accumulation of FhuF is negligible in Salmonella grown in LB and iron-supplemented LB (Fig. 2b, lanes 1 and 3), but increases in bacteria cultivated in the presence of 2,2'-bipyridyl or UHA (Fig. 2b, lanes 4–6). In both cases, protein accumulation is proportional to UHA concentrations. The aminopeptidase PepT, which is known to be positively regulated by Fur, is present at slightly higher levels in iron-rich conditions (Fig. 2c, lanes 1 and 3) and decreases in the presence of 2,2'-bipyridyl or high concentrations of UHA (Fig. 2c, lanes 2 and 6). In contrast, the SitA protein, which is known to be negatively regulated by Fur, is repressed in the presence of FeCl$_3$ (Fig. 2d, lane 3) but is insensitive both to 2,2'-bipyridyl and UHA (lanes 2 and 4–6), showing the same accumulation pattern in standard LB medium (lane 1) and in the media supplemented with these agents. These results suggest a hierarchy in the expression of Fur-regulated genes, sitA being expressed at different iron concentrations than those required to activate iroB and fhuF.

Our findings indicate that three of the four Fur-regulated genes we have analysed are responsive to UHA. Up- and downregulation of these genes suggests that UHA effectively decreases iron availability for Salmonella and is consistent with the hypothesis that UHA compromises iron availability for the microorganism. In contrast, treatment of bacteria with UDA did not induce IroB accumulation (Fig. 3). This finding demonstrates that the mechanisms of action of UDA and UHA are not completely overlapping and suggest that the introduction in UHA of a hydroxamic group able to chelate iron actually disturbs iron homeostasis in Salmonella and, most likely, in all the microorganisms that show a high sensitivity to UHA.

To verify the specific ability of UHA to influence the expression of genes involved in iron homeostasis, we have analysed the accumulation profiles of ZnuA, the periplasmic component of a high-affinity zinc transporter coded by the znuABC operon. This target was chosen because expression...
of znuABC is under the transcriptional control of Zur, a Fur-like metalloregulatory DNA-binding protein highly responsive to small variations in the intracellular concentration of zinc [Ammendola et al. (2007) and references therein]. Salmonella strains SA213 and SA140 (see Table 2) were grown overnight in LB, diluted 1 : 500 in LB supplemented with 2,2'-bipyridyl or increasing concentrations of UHA and grown for 18 h at 37 °C. Western blot analysis of bacterial lysates indicates that whereas IroB is induced in the presence of UHA, ZnuA is not affected by iron availability, being expressed at the same level in all the conditions tested (data not shown).

In conclusion, our data demonstrate that UHA is a novel antimicrobial agent, more effective that UDA in controlling the growth of a number of medically important microorganisms, combining a hydrophobic nature that permits an efficient binding to membranes with an iron-chelating activity, which likely decreases microbial growth by interfering with intracellular iron availability. These findings provide a novel example of the potential efficacy of antimicrobial approaches based on metal-chelating agents and suggest a novel strategy to improve the therapeutic efficacy of fatty acids.

Acknowledgements

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Sigle H-C, Theuwes S, Niewerth M, Korting HC, Schafer-Korting M & Hube B (2005) Oxygen accessibility and iron levels are...


### Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** $^1$H-NMR of UHA.

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