Mechanistic Assessment of DNA Ligase as an Antibacterial Target in *Staphylococcus aureus*

Steven D. Podos, Jane A. Thanassi, and Michael J. Pucci*

Achillion Pharmaceuticals, New Haven, CT 06511

Running title: DNA Ligase as an antibacterial target

*Corresponding author

Mailing address:

Achillion Pharmaceuticals
300 George Street
New Haven, CT 06511

Phone: (203) 624-7000

Fax: (203) 624-7003

E-mail: mpucci@achillion.com
We report the use of a known pyridochromanone inhibitor with antibacterial activity to assess the validity of NAD⁺-dependent DNA ligase (LigA) as an antibacterial target in *Staphylococcus aureus*. Potent inhibition of purified LigA was demonstrated in a DNA ligation assay ($K_i = 4.0$ nM) and in a DNA-independent enzyme adenylation assay using full-length LigA ($IC_{50} = 28$ nM) or its isolated adenylation domain ($IC_{50} = 36$ nM). Antistaphylococcal activity was confirmed against MSSA and MRSA strains (MIC = 1.0 $\mu$g/ml). Analysis of spontaneous resistance potential revealed a high frequency emergence ($4 \times 10^{-7}$) of high level resistant mutants (MIC > 64) with associated ligA lesions. There were no observable effects on growth rate in these mutants. Of 22 sequenced clones, three encoded point substitutions within the catalytic adenylation domain and 19 in the downstream OB-fold and helix-hairpin-helix (HhH) domains. *In vitro* characterization of the enzymatic properties of four selected mutants revealed distinct signatures underlying their resistance to inhibition. The infrequent adenylation domain mutations altered the kinetics of adenylation and likely elicited resistance directly. In contrast, the highly represented OB-fold domain mutations demonstrated a generalized resistance mechanism in which covalent LigA activation proceeds normally yet the parameters of downstream ligation steps are altered. A resulting decrease in substrate $K_m$ and a consequent increase in substrate occupancy renders LigA resistant to competitive inhibition. We conclude that the observed tolerance of staphylococcal cells to such hypomorphic mutations likely invalidates LigA as a viable target for antistaphylococcal chemotherapy.
INTRODUCTION

NAD⁺-dependent DNA ligase (LigA) has been identified by numerous authors as an attractive potential target for broad-spectrum antibacterial chemotherapy (7, 24). LigA is well conserved among eubacterial species, is architecturally and biochemically distinct from the ATP-dependent DNA ligases of eukaryotic cells, and has been found to be essential for bacterial viability wherever examined (13, 14, 15, 17, 32). Moreover, the DNA ligation reaction has been dissected mechanistically, mutationally, and structurally (8, 20, 26, 27, 34, 35, 36), and screening assays have been reported for the complete reaction cycle and for individual component steps (2, 11, 18).

DNA ligation activities are essential for multiple DNA processes in replication and repair, including the joining of Okazaki fragments into a continuous strand during chromosomal DNA replication. Enzymatically, DNA ligation proceeds via three successive adenylyl transfer steps (Fig. 1) (33): first, DNA-independent covalent adenylation of the catalytic lysine by the NAD⁺ substrate; second, adenylyl transfer to the free 5’ phosphate at the nicked DNA ligation site; and third, the covalent sealing of the DNA nick with concomitant AMP release. Biochemical functions of distinct domains in the modular enzyme structure have been assigned to particular reaction steps. The DNA-independent adenylyl transfer activity resides within the amino-terminal adenylation domain, which comprises an amino-terminal Ia region that is specific to NAD⁺-dependent DNA ligases and a nucleotidyl transferase (NTase) region that is universal among DNA and RNA ligases. The subsequent coupling of adenylation to DNA ligation depends upon downstream DNA-binding domains which include an oligonucleotide-binding fold (OB-fold) and a helix-hairpin-helix (HhH) domain. Structural studies of the adenylation
domain have revealed conformational transitions that accompany the adenylation cycle (8), and structural study of the full-length enzyme bound to DNA-adenylate has identified specific contacts between the DNA-binding domains and the DNA duplex substrate near the nicked ligation site (20).

Numerous LigA inhibitors have been reported to date including arylamino acids such as chloroquine (3); glycosyl ureides and glycosylamines (28, 29); tetracyclic indoles (30); a pyrimidopyrimidine inhibitor (17); substituted adenosine analogs (19, 31); and the pyridochromanones (1). Pyridochromanones were identified by high-throughput screening as potent competitive inhibitors of DNA ligation by LigA from Staphylococcus aureus (IC$_{50}$ ≤ 0.9 μM) (1). They inhibit LigA from diverse bacteria but are inactive against the ATP-dependent human DNA ligase I (1, 9). Moreover they show antibacterial activity against S. aureus (MIC ≤ 1 μg/ml), with a bactericidal mode of action; their antibacterial activity in S. aureus has been mapped by a putative resistance lesion to the ligA locus.

In this study we applied the antibacterial activity of a pyridochromanone inhibitor to assess LigA as an antibacterial target in S. aureus. We report the recovery of numerous high-level resistance mutations dispersed across the ligA gene, with an unexpected concentration of mutations in the OB-fold domain. We examined the kinetic parameters of several mutant LigA isoforms and report a generalized resistance mechanism in which LigA resistance to competitive inhibitors is achieved via systematic alteration of its kinetic properties. The facility of this mechanism, coupled with the tolerance of the bacteria to broad changes in LigA properties, suggests that LigA makes a poor antibacterial drug target despite its favorable features. Assessment of this potential
antibacterial target therefore requires greater subtlety than afforded by standard validation
criteria.
MATERIALS AND METHODS

Bacterial strains and compounds. *S. aureus* ATCC 29213 (methicillin-sensitive; MSSA), *S. aureus* ATCC 700699 (methicillin-resistant; MRSA), and *E. coli* ATCC 25922 were obtained from the American Type Culture Collection, Manassas, VA. Pyridochromanone compounds 1 and 2 were synthesized at Achillion Pharmaceuticals. Adenosine 3′, 5′-cyclic monophosphorothioate (Sp-cAMPs) was purchased from Sigma-Aldrich, St. Louis, MO.

Culture conditions. *S. aureus* strains were grown with aeration at 35°C-37°C in Mueller Hinton II (MH II) broth (Becton Dickinson, Sparks, MD).

Compound susceptibility assays. MICs were determined by broth microdilution according to the CLSI approved guidelines (5).

Selection and characterization of resistant mutants. *S. aureus* ATCC 29213 cultures were grown overnight under non-selective conditions in BHI or MH II liquid media. Spontaneous pyridochromanone-resistant mutants were recovered from these cultures by plating ~1 x 10⁹ organisms followed by overnight growth at 37°C on BHI or MH II agar containing 4 μg/ml compound 1. Recovered clones were assessed for compound susceptibility and *ligA* genotype. Genomic *ligA* DNA was amplified by PCR using the primers SaLig-N (ATATACCATGGCTGATTTATCGTCTCGTG, NcoI restriction site underlined) and SaLig-C (TTATATAAGCGGCCGACTATTTAATTCTTTGCTTATCTACA, NotI restriction site underlined). Genomic *ligA* products were purified by QIAquick PCR Purification (QIAGen, Valencia, CA) and their coding sequences determined by...
automated DNA sequencing (W.M. Keck Foundation, Yale University, New Haven, CT, and SeqWright, Houston, TX).

**LigA protein expression and purification.** *ligA* was amplified by PCR from *S. aureus* strain N315 with the primers SaLig-N and SaLig-C and the product cloned into the NcoI and NotI sites of the pET21d vector (Novagen) to direct the expression of LigA with a C-terminal His-tag. Missense mutants and the carboxy-terminally tagged LigA truncation (LigA-Δ comprising residues 1-315) were introduced by QuikChange site-directed mutagenesis according to the manufacturer’s instructions (Stratagene, La Jolla, CA). All isoforms were expressed in *E. coli* BL21(DE3)pLysS (Novagen EMD Biosciences, Madison, WI), purified by HisTrap or HisGraviTrap chromatography (GE Healthcare, Piscataway, NJ), and de-adenylated by incubation with excess NMN and MgCl₂ followed by dialysis.

**LigA adenylation assays.** 10 nM LigA was incubated with 1 nM [³²P-AMP]-NAD⁺ in buffer A (10 mM HEPES, 25 mM KCl, 20 mM MgCl₂, 1 mM DTT, 10% PEG 8000, pH = 8.0). Reactions were stopped by EDTA after incubation periods ranging from 7.5 to 60 min, and [³²P]-AMP incorporation into LigA was assessed by SDS-PAGE or by a MultiScreen DE or IP filter binding assay (Millipore, Billerica, MA) similar to that of Miesel et al (18) followed by scintillation counting in a Wallac MicroBeta reader (PerkinElmer, Waltham, MA). Reaction rates in the filter-binding assay were converted from cpm to pM units using the empirically determined specific activity of [³²P]-NAD⁺ substrate.
DNA ligation assays. The oligonucleotides 1 through 4 were synthesized by IDT (Coralville, IA) as defined by Chen et al (2) with the duplex pairs 1-2 and 3-4 bearing complementary 4 bp overhangs. 1 nM LigA was incubated in buffer A with 10 μM NAD⁺ and 1.25 ng/μl oligonucleotide duplex DNAs 1-2 and 3-4. Reactions were incubated for 10-12 min (wild-type LigA) or 40 min (mutant LigA) to optimize signal while keeping reactions within linear range; reactions were terminated by EDTA addition. Ligation was assessed by electrophoresis through 15% polyacrylamide gels (Bio-Rad, Hercules, CA) in Tris/boric acid/EDTA buffer followed by SYBR gold staining (Invitrogen, Carlsbad, CA) and photographic quantitation (Alpha Innotech, San Leandro, CA).

Kinetic analyses. Reactions were conducted in the absence or presence of test compound. Kinetic parameters were calculated by non-linear regression methods using Prism software (GraphPad, San Diego, CA). Parameters include: $V_o$ for LigA adenylation reactions as derived from relative reaction rates; $K_m$ and $k_{cat}$ for DNA ligation at varying NAD⁺ concentrations; $IC_{50}$ for adenylation and ligation reactions in the presence of inhibitory compound concentration series; and $K_i$ for the ligation reaction catalyzed by the wild-type enzyme. $K_i$ was also determined for the ligation reaction catalyzed by the wild-type enzyme by the Cheng-Prusoff equation $K_i = IC_{50} / (1 + [S] / K_m)$ (3).
RESULTS

**Inhibition of DNA ligation and LigA adenylation by pyridochromanones.** To explore the validity of LigA as an antibacterial target, we examined its susceptibility to two pyridochromanone inhibitors (Fig. 2). We confirmed that these compounds have antibacterial activity against MSSA and MRSA strains of *S. aureus* (MIC = 1 µg/ml for both strains) but not against *E. coli* (Table 1). We also confirmed that these compounds are potent *in vitro* inhibitors of DNA ligation catalyzed by *S. aureus* LigA (Table 1), and we determined that these compounds inhibit the DNA-independent auto-adenylation activity of both full-length LigA and a truncated enzyme LigA(1-315) comprising the isolated adenylation domain (Table 1). This inhibition of LigA auto-adenylation establishes directly the adenylation domain as the locus of pyridochromanone inhibition, consistent with previous suggestions and our finding (Table 1) that these compounds compete for NAD⁺ substrate binding.

**Pyridochromanone resistance mutations dispersed throughout ligA.** To characterize the mechanism of pyridochromanone action in *S. aureus*, 22 resistant derivatives of MSSA strain 29213 were collected from 13 independent cultures following growth on agar plates under selection by compound 1 at 4× MIC (Table 2). Resistant mutants were observed to arise at a frequency of 4×10⁻⁷. All recovered mutants showed high-level resistance, as MIC values for compound 1 were elevated > 64-fold above that of the parental strain (Table 2). All resistant colonies appeared phenotypically normal, and three showed normal doubling times when measured in liquid culture (ACH-0342, ACH-0343, and ACH-0344, not shown).
Missense point mutations were identified within the *ligA* gene in all 22 resistant colonies, establishing *ligA* as the immediate target of compound 1 activity (Table 2). Surprisingly the mutations were dispersed throughout the *ligA* gene (Fig. 3). Only three were located within the adenylation domain, despite the demonstrated localized activity of compound 1 upon this domain. The remaining 19 were located in the DNA-binding OB-fold and HhH domains, with the majority in the OB-fold domain similar to the previously reported resistance mutation Ala\(^{373}\) (1). The concentration of lesions in these downstream domains suggests a facile resistance pathway whereby mutations in the DNA-binding domains can overcome pyridochromanone inhibition in the adenylation domain, such as by altering the conformational landscape of the enzyme or its interaction with DNA, yet without compromising the essential functions of this enzyme in bacterial replication.

**Kinetic properties defining three functional classes of LigA mutant.** To dissect the mechanism underlying high-level pyridochromanone resistance, we engineered four of the observed resistance mutations individually into the full-length LigA enzyme (Fig. 4A). Arg\(^{61}\)Ile and Ala\(^{303}\)Asp were chosen to represent the relatively uncommon adenylation domain mutations; Ala\(^{349}\)Val and Ala\(^{373}\)Thr represented the more common OB-fold domain mutations that likely confer pyridochromanone resistance from a distance. All four LigA mutant variants were expressed, purified, and subjected to enzymological study.

First we examined the NAD\(^+\)-dependent but DNA-independent LigA adenylation step that pre-activates the enzyme (Fig. 4B, Table 3). LigA is consumed as a substrate during this single-turnover reaction, which therefore is not amenable to classic
enzymological analysis. We reduced NAD$^+$ concentration to 1 nM, far below its
expected $K_m$, to slow this reaction sufficiently for measurement of adenylation rates. The
two adenylation domain mutations had opposite effects on the adenylation rate, as the
Arg$^{61}$Ile lesion effected a 15-fold increase in rate whereas the Ala$^{303}$Asp lesion conferred
a 7-fold decrease. In contrast the two OB-fold mutations did not affect the adenylation
rate, consistent with their locations apart from the adenylation domain.

Next we examined the Michaelis-Menten kinetics of the full DNA ligation
reaction using an oligonucleotide substrate and a range of NAD$^+$ concentrations (Fig. 4C,
Table 4). All four LigA mutations caused 6.0-fold to 14-fold reductions in maximal
ligation rate, as represented by $k_{cat}$, but also 9.1-fold to > 69-fold reductions in NAD$^+$ $K_m$.
Therefore, the DNA ligation reaction is not much affected at low NAD$^+$ concentration, as
evidenced by the relatively modest impacts of the lesions on catalytic efficiency $k_{cat} / K_m$,
but is significantly compromised at higher NAD$^+$ concentrations.

These LigA mutations therefore define three functional classes according to their
kinetic properties, in which DNA-independent pre-activation of enzyme (LigA
adenylation) is accelerated, hindered, or unaffected, yet the parameters of the complete
reaction cycle (DNA ligation) are significantly compromised in all cases. Each class
likely represents a distinct mechanism by which LigA is altered conformationally or
energetically to produce enzyme that retains sufficient activity for viability yet has
acquired resistance to active site inhibition.

**Resistance of mutant LigA enzymatic activities to inhibition.** Finally we
examined the impacts of the three classes of resistance mutations on LigA susceptibility
to compound 1 inhibition in the enzyme reaction assays (Fig. 5A, Table 4). In the LigA adenylation reaction, the Arg<sup>61</sup>Ile lesion caused minimal elevation in IC<sub>50</sub> (2.1-fold) whereas Ala<sup>303</sup>Asp increased the IC<sub>50</sub> by > 36-fold. The OB-fold domain mutations Ala<sup>349</sup>Val and Ala<sup>373</sup>Thr as expected had no effect on inhibition of the adenylation reaction by compound 1. We conclude that only the Ala<sup>303</sup>Asp lesion significantly affects the affinity between LigA and the pyridochromanone inhibitor.

In the DNA ligation reaction, in contrast, all four LigA lesions conferred significant resistance to pyridochromanone inhibition (Fig. 5B, Table 4). IC<sub>50</sub> values for compound 1 were elevated by > 110-fold for the two adenylation domain mutants and 18-fold and 35-fold for the two OB-fold domain mutants, accounting for the antibacterial resistance phenotypes of the corresponding mutant staphylococci. Mutant LigA was similarly resistant to the ATP analog Sp-cAMPs which competes for access to active site of the adenylation domain (Table 4) (18). The elevated IC<sub>50</sub> values against the two OB-fold LigA mutants in the DNA ligation assay were not accompanied by corresponding increases in K<sub>i</sub> values. Together, these findings support the view that the majority of LigA mutations confer resistance without direct changes in inhibitor binding at the active site, instead altering active site accessibility by affecting the downstream steps of the three-step DNA ligation reaction.
DISCUSSION

We have re-examined the potential of NAD\(^+\)-dependent DNA ligase (LigA) as an antibacterial target in *S. aureus*. We recovered 22 mutants with *ligA* coding lesions that were highly resistant to a known pyridochromanone inhibitor yet showed no deficits in viability or growth. Three lesions were recovered within the adenylation domain; the remaining 19 were located within the DNA-binding OB-fold (16 of 22) and HhH (3 of 22) DNA-binding domains. Although the possibility of distal lesions was anticipated by the prior resistance mutation (1), their predominance was unexpected as we have established the adenylation domain as the focus of pyridochromanone action. Our enzymological analysis of four representative mutant LigA isoforms has identified three mechanistic classes of resistance. We suggest that the observed tolerance of staphylococci to resistance mutations, despite the altered enzymology of DNA ligation, presents a general resistance pathway that renders LigA a questionable antibacterial target despite its favorable features.

The adenylation domain mutations Arg\(^{61}\)Ile and Ala\(^{293}\)Asp exert opposing effects on the rate of DNA-independent LigA adenylation yet cause comparable reductions in the catalytic rate for DNA ligation. These effects can be illuminated by examination of the available crystal structures of LigA adenylation domains from several pathogenic bacteria, some featuring bound ligands including substrate, product, and/or various inhibitors (Fig. 6A) (8, 12, 19, 23, 28). Most structures show an “open” orientation of domain Ia relative to the NTase domain, with two well-separated (>\(20\) Å) ligand-binding sites at the domain Ia surface and the NTase active site. In contrast, the binding of NAD\(^+\) substrate directs the large-scale reorientation of domain Ia about a hinge region to enclose...
NAD\(^+\) within the active site, now in contact with both ligand-binding sites, and define a “closed” orientation (8).

The Arg\(^{61}\)Ile mutation alters a well-conserved residue near this hinge region (Fig. 6A). This proximity suggests that the mutation might elicit the observed changes in LigA kinetics by favoring the closed orientation upon NAD\(^+\) binding, such as from the loss of interactions seen between Arg\(^{61}\) in the open orientation with NTase surface moieties including the backbone carbonyl of Ile\(^{152}\). An accelerated LigA closing would increase the LigA adenylation rate as observed, and a disfavored return to the open conformation could also cause the observed retardation of DNA-dependent steps such as DNA ligation and/or release. The latter could also increase active site occupancy by the adenylate product and thus confer reduced susceptibility to pyridochromanones and other competitive inhibitors.

The Ala\(^{303}\)Asp mutation in contrast alters a well-conserved residue underlying the catalytic region, abutting the residues Leu\(^{82}\) and Val\(^{281}\) that contact NAD\(^+\) substrate (Fig. 6A) (8). This mutation was observed to lower the catalytic rates of LigA adenylation and DNA ligation, without lowering catalytic efficiency of ligation, suggesting that the alteration diminishes active-site function without affecting the initial NAD\(^+\) binding. Additionally, the proximity of Ala\(^{303}\) to bound pyridochromanone compound 1 (5.3 Å) (23) suggests that mutation could directly alter the inhibitor binding interface to generate the observed high-level pyridochromanone resistance.

The high-frequency lesions within the DNA-binding OB-fold domain define a third mechanistic class of resistance mutation, as the test mutations Ala\(^{349}\)Val and...
Ala^{373}Thr do not alter the kinetics of LigA adenylation yet still cause significant decreases in $K_m$ and $k_{cat}$ values for DNA ligation. These results highlight the importance of the DNA-binding domains in coordinating the adenylyl transfer steps with the DNA-dependent steps of ligation.

The reported crystal structure of *E. coli* LigA bound to nicked adenylated DNA shows the OB-fold and HhH domains in numerous contacts with the enveloped DNA duplex (Fig. 6B) (20). The OB-fold domain contacts DNA at several of the residues identified as resistance loci: Arg^{326} forms a hydrogen bond with the DNA backbone; Ala^{349} and Ala^{373} directly abut the DNA backbone; His^{352} extends into the DNA minor groove; and, Pro^{332} and Leu^{351} are adjacent to contact sites and likely influence the structure of the binding surface (numbering from *S. aureus*). The HhH domain also forms extensive contacts with bound DNA via four hexapeptide “loop-helix” motifs, each including an internal glycine that initiates the helix and creates an oxyanion hole that accepts a backbone phosphate. The resistance loci Gly^{481} and Gly^{585} define two such “loop-helix” glycines, and Ala^{549} resides within a “loop-helix” helix and also likely contributes to DNA binding.

The proximity of these lesions to DNA contact sites suggests that they reduce the catalytic rates for DNA ligation by lowering the affinity for DNA. We propose that these lowered catalytic rates can explain entirely the observed decreases in $K_m$ values and, most significantly, the high-level resistance to inhibition. First, by stalling the DNA ligation reaction, these lesions could stabilize the adenylated LigA intermediate and thus lower substrate $K_m$ values as observed. Second, this increased active site occupancy would also necessarily confer resistance to pyridochromanone compounds and other competitive inhibitors.
inhibitors. In this view, mutations in the distal domains can elicit high-level resistance as a secondary consequence of altered reaction kinetics.

The ligA gene is essential in every bacterial species examined to date including S. aureus (13, 32). In this study, however, we recovered a series of highly pyridochromanone-resistant ligA mutants of S. aureus with no obvious growth defects despite dramatic deficits in DNA ligation kinetics. These results indicate that a significant portion of LigA function is dispensable for viability, and that its modular composition facilitates ready diminishment by distal mutations that confer inhibitor resistance while leaving sufficient residual activity for viability. These results challenge the validity of LigA as a suitable target for antibacterial therapy and emphasize the need for subtler considerations of its biological requirement in staphylococci.

Classical genetic studies had previously indicated that E. coli can tolerate significant losses of LigA activity, as the conditional lethal mutant ligts7 and the viable mutant lig4 show 10- to 20-fold reductions in Okazaki fragment joining even under permissive growth conditions (10, 16). A complementation study in Mycobacteria has also suggested that significant LigA depletion has little effect on growth and viability (14). Our resistance study in S. aureus has confirmed that LigA has a large margin for variation with which to escape inhibitor action. The apparent excess of LigA activity in wild-type cells might be due to its multiple roles: low concentrations are sufficient for essential replicative functions yet higher concentrations are available for repair functions.

The difficulty in leveraging target enzymes into viable antibacterial therapies has been much discussed recently (21, 22, 25). Further study will be required to determine
the true utility of LigA as a target for standard inhibitors, or whether rare inhibitors might
be required that kill via gain-of-function “poison” mechanism analogous to the DNA
breakage induced by quinolones. (6). In summation, our study highlights the importance
of a more thorough analysis of LigA target validity for antibacterial drug discovery.

ACKNOWLEDGMENTS

We acknowledge Akihiro Hashimoto, Godwin Pais, and Barton Bradbury for synthetic
chemistry efforts and Christy Thoma and Jijun Cheng for technical support.
REFERENCES


### TABLE 1. Potency of pyridochromanones against bacterial test strains and *S. aureus* LigA enzymatic activities.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MIC (μg/ml)</th>
<th><em>K</em> (nM)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>S. aureus</em> ATCC 29213</td>
<td><em>S. aureus</em> ATCC 700699</td>
<td><em>E. coli</em> ATCC 25922</td>
</tr>
<tr>
<td>Compound 1</td>
<td>1</td>
<td>1</td>
<td>&gt; 64</td>
</tr>
<tr>
<td>Compound 2</td>
<td>1</td>
<td>1</td>
<td>&gt; 64</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.25</td>
<td>64</td>
<td>0.016</td>
</tr>
</tbody>
</table>

<sup>a</sup> *K* for compound 1 against full-length LigA was determined by non-linear regression analysis using a mixed-competitive model. Inhibition was found in this analysis to be partly competitive (α = 3.2).

<sup>b</sup> LigA:AD is truncated after residue 315. The adenylation domain is included, comprising domain Ia and the NTase region, but the downstream DNA-binding domains are omitted.

<sup>c</sup> LigA:FL refers to the full-length LigA enzyme.

<sup>d</sup> ND = Not determined.

### TABLE 2. *S. aureus* mutants with resistance to Compound 1.

<table>
<thead>
<tr>
<th>Strain</th>
<th>LigA substitution</th>
<th>MIC (μg/ml)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Strain</th>
<th>LigA substitution</th>
<th>MIC (μg/ml)&lt;sup&gt;c&lt;/sup&gt;</th>
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<td>wild-type</td>
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<td>Parent&lt;sup&gt;d&lt;/sup&gt;</td>
<td>wild-type</td>
<td>1</td>
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<td>ACH-0275</td>
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<td>ACH-0341</td>
<td>Gly&lt;sup&gt;545&lt;/sup&gt;Val</td>
<td>&gt; 64</td>
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<td>ACH-0342</td>
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<tr>
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<td>&gt; 64</td>
<td>ACH-0343</td>
<td>Arg&lt;sup&gt;61&lt;/sup&gt;lle</td>
<td>&gt; 64</td>
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<td>&gt; 64</td>
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<td>&gt; 64</td>
<td>ACH-0350</td>
<td>Ala&lt;sup&gt;349&lt;/sup&gt;Val</td>
<td>&gt; 64</td>
</tr>
</tbody>
</table>

<sup>a</sup> Selection I: 10 colonies resistant colonies were isolated from a single culture.

<sup>b</sup> Selection II: 12 independent resistant colonies were obtained from twelve separate cultures.

<sup>c</sup> Antibacterial activity of compound 1 against parent or mutant as represented by MIC.

<sup>d</sup> *S. aureus* ATCC 29213.
TABLE 3. Activities of wild-type and mutant LigA isoforms.

<table>
<thead>
<tr>
<th>LigA isoform</th>
<th>$V_o$ (pM·min$^{-1}$)</th>
<th>$k_{cat}$ (normalized)$^c$</th>
<th>$K_m$ (μM NAD$^+$) (normalized)$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>LigA</td>
<td>1.9 ± 0.10</td>
<td>1.0</td>
<td>5.4 ± 0.26</td>
</tr>
<tr>
<td>LigA:AD</td>
<td>1.3 ± 0.023</td>
<td>NA$^e$</td>
<td>NA</td>
</tr>
<tr>
<td>LigA(Arg$^{61}$Ile)</td>
<td>28 ± 1.9</td>
<td>0.077 ± 0.0043</td>
<td>&lt;0.078</td>
</tr>
<tr>
<td>LigA(Ala$^{503}$Asp)</td>
<td>0.29 ± 0.015</td>
<td>0.075 ± 0.0035</td>
<td>0.59 ± 0.042</td>
</tr>
<tr>
<td>LigA(Ala$^{495}$Val)</td>
<td>1.9 ± 0.0036</td>
<td>0.070 ± 0.0021</td>
<td>0.92 ± 0.0082</td>
</tr>
<tr>
<td>LigA(Ala$^{737}$Thr)</td>
<td>1.9 ± 0.0070</td>
<td>0.17 ± 0.012</td>
<td>0.13 ± 0.014</td>
</tr>
</tbody>
</table>

$^a$ $V_o$ = adenylation rate as depicted in Fig. 4B. Mean ± SEM from n = 2 experiments.

$^b$ Kinetic parameters for the complete DNA ligation reaction as depicted in Fig. 4C. Mean ±SEM from n = 4 (wild-type) or n = 2 (mutants) experiments.

$^c$ $k_{cat}$ was determined for each isoform in relative units (relative fluorescence intensity of product generated per unit time and unit enzyme) and then normalized to wild-type LigA.

$^d$ $k_{cat}/K_m$ was normalized for each isoform to wild-type LigA in each experiment as described above in footnote c.

$^e$ NA = Not applicable.

TABLE 4. Inhibition of wild-type and mutant LigA adenylation and DNA ligation activities.

<table>
<thead>
<tr>
<th>LigA isoform</th>
<th>Compound 1 $I_{C_{50}}$ (nM)$^a$</th>
<th>$K_i$ (nM)$^b$</th>
<th>$K_{cat}/K_m$ (μM)$^c$</th>
<th>$K_{cat}/K_m$ (μM)$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>LigA</td>
<td>28 ± 1.1</td>
<td>9.0 ± 0.48</td>
<td>3.1</td>
<td>1.5 ± 0.03</td>
</tr>
<tr>
<td>LigA:AD</td>
<td>36 ± 1.5</td>
<td>NA$^e$</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>LigA(Arg$^{61}$Ile)</td>
<td>58 ± 1.3</td>
<td>&gt;1000</td>
<td>ND$^d$</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>LigA(Ala$^{503}$Asp)</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;56</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>LigA(Ala$^{495}$Val)</td>
<td>25 ± 1.7</td>
<td>320 ± 27</td>
<td>2.7</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>LigA(Ala$^{737}$Thr)</td>
<td>28 ± 0.66</td>
<td>160 ± 7.1</td>
<td>2.2</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

$^a$ $I_{C_{50}}$ of inhibitor in LigA adenylation and DNA ligation reactions as depicted in Fig. 5. Mean ± SEM from n = 2 – 3 experiments.

$^b$ $K_i$ for DNA ligation reaction, as calculated according to the Cheng-Prusoff equation: $K_i = I_{C_{50}} / (1 + [S] / K_m)$, where [S] = 10 μM NAD$^+$ and $K_m$ is taken from Table 3.

$^c$ NA = Not applicable.

$^d$ ND = Not determined: $K_i$ could not be calculated from available data.
FIGURE LEGENDS

FIG. 1. Reaction scheme depicting the three successive adenylyl transfer steps that underlie the DNA ligation reaction catalyzed by eubacterial NAD⁺-dependent DNA ligase (LigA). (A) Step 1, DNA-independent adenylation of the catalytic lysine of LigA (depicted as transfer of pA), using NAD⁺ (NppA) as substrate and releasing NMN (pN) product. (B) Step 2, covalent transfer of AMP (pA) from LigA to the 5’ phosphate of target DNA strand b. (C) Step 3, ligation of DNA strands a and b with release of AMP from DNA strand b. (A) through (C): for simplicity the single DNA strands a and b are depicted without complementary DNA; gray arrows indicate electron movements during the successive adenylyl transfers.

FIG. 2. Pyridochromanone inhibitors. Compounds 1 and 2 correspond to compounds 2 and 3 respectively of Brötz-Oesterhelt et al (1).

FIG 3. Dispersed distribution of pyridochromanone resistance mutations. Modular architecture of the bacterial LigA enzyme is indicated: Ia, N-terminal NAD⁺-specific portion of the adenylation domain (S. aureus amino acids 1-69); NTase, nucleotidyl transferase portion of the adenylation domain (70-309); OB, oligonucleotide-binding fold domain (310-397); ZF, zinc finger domain (398-424); HhH, helix-hairpin helix domain (425-578); BRCT, BRCA C-terminal domain (580-667). ligA lesions identified in this study are indicated (arrows); the four mutations subjected to enzymological analysis in this study, comprising three identified here and Ala373 of
Brötz-Oesterhelt et al (1), are further indicated by black arrows and amino acid designations.

FIG. 4. Enzymological properties of mutant LigA. (A) Purified wild-type and mutant LigA visualized by SDS-PAGE and Coomassie staining. (B) Adenylation time-course with 10 nM LigA and 1 nM [³²P]-NAD⁺. Mean ± SEM are shown for the duplicate wells of a single representative experiment. (C) DNA ligation reactions catalyzed by 1 nM LigA at varying NAD⁺ concentrations. Data points indicate reaction rates from a single representative experiment; curves indicate Michaelis-Menten curves as fitted by non-linear regression analysis. Wild-type and mutants are plotted on separate panels because of their differing NAD⁺ concentration scales. Symbols: ●, LigA; *, LigA(1-315) (B only); △, LigA(Arg⁶¹Ile); ∇, LigA(Ala³⁰³Asp); ◇, LigA(Ala³⁴⁹Val); □, LigA(Ala³⁷³Thr).

FIG 5. Resistance of LigA mutants to pyridochromanone inhibition. (A) Inhibition by compound 1 of adenylation reactions conducted with 10 nM LigA and 1 nM [³²P]-NAD⁺. (B) Inhibition by compound 1 of DNA ligation reactions catalyzed by 1 nM LigA with 10 μM-NAD⁺. (A) and (B): Data points indicate normalized reaction rates from a single representative experiment; error bars in (A) represent error among duplicate wells. Curves indicate sigmoidal inhibition curves as fitted by nonlinear regression. Symbols: ●, LigA; *, LigA:AD; △, LigA(Arg⁶¹Ile); ∇, LigA(Ala³⁰³Asp); ◇, LigA(Ala³⁴⁹Val); □, LigA(Ala³⁷³Thr).
FIG 6. Structural consideration of the three classes of resistance mutation. (A)

Overlaid LigA adenylation domain structures showing aligned open and closed orientations of domain Ia (gold) relative to the NTase (blue). The overlay comprises three Enterococcus faecalis structures with bound ligands including two open conformations (1TA8 with bound NMN; 3AB with bound NMN and pyridochromanone compound 1) and one closed conformation (1TAE, bound NAD\(^+\), and one S. aureus structure in the open conformation (3JSL apoenzyme, white strand (8, 12, 23)). Side chains are shown for the adenylation domain resistance loci Arg\(^{61}\) (yellow) and Ala\(^{303}\) (magenta) and for the catalytic amino acid Lys\(^{112}\) (cyan). Ligands: NMN bound to domain Ia in open conformation (indigo); compound 1 bound to active site in open conformation (red); and NAD\(^+\) bound to active site in closed conformation (green). Arrow, pivot point at conformational hinge for domain Ia reorientation. (B) Modular structure of E. coli LigA bound to nicked adenylated DNA (2OWO) (20) with resistance loci indicated for the OB-fold and HhH domains. Left panel, complete structure; center panel, OB-fold with proximate DNA base pairs; right panel, HhH domain with proximate DNA base pairs. OB-fold domain (green), resistance loci are shown with side chains (indigo); HhH domain (yellow), resistance loci are shown with side chains or, for glycine residues, marked by asterisks (red). All numbering refers to the S. aureus LigA sequence.
Compound 1

Compound 2