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Staphylococcus aureus MurC Participates in L-Alanine Recognition *via* Histidine 343, a Conserved Motif in the Shallow Hydrophobic Pocket

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UDP-*N*-acetylmuramic acid:L-alanine ligase that is encoded by the *murC* gene, is indispensable for bacterial peptidoglycan biosynthesis and an important target for the development of antibacterial agents. Structure of MurC ligase with substrates has been described, however, little validation *via* studying the effects of mutations on the structure of MurC has been performed. In this study, we carried out a functional *in vitro* and *in vivo* characterization of *Staphylococcus aureus* MurCH343Y protein that has a temperature-sensitive mutation of a conserved residue in the predicted shallow hydrophobic pocket that holds a short L-alanine side chain. Purified H343Y and wild-type MurC had K_m values for L-alanine of 3.2 and 0.44 mM, respectively, whereas there was no significant difference in their K_m values for ATP and UDP-*N*-acetylmuramic acid, suggesting the specific alteration of L-alanine recognition in MurCH343Y protein. In a synthetic medium that excluded L-alanine, *S. aureus murCH343Y* mutant cells showed an allele-specific slow growth phenotype that was suppressed by addition of L-alanine. These results suggest that His343 of *S. aureus* MurC is essential for high-affinity binding to L-alanine both *in vitro* and *in vivo* and provide experimental evidence supporting the structural information of MurC ligase.

Key words: Gram-positive bacteria, MurC, peptidoglycan, *Staphylococcus aureus*, UDP-*N*-acetylmuramic acid:L-alanine ligase.

Abbreviations: UDP-MurNAc, UDP-*N*-acetylmuramic acid.

Peptidoglycans are components of the bacterial cell wall that confer mechanical resistance to counteract the higher internal osmotic pressure and maintain a defined cell shape. The mesh-like structure of peptidoglycans consists of a sheet of glycan chains connected by short cross-linking peptides, in which disaccharides with short peptides constitute a single unit. The enzymes involved in peptidoglycan biosynthesis are restricted to bacteria and thus, are very attractive targets for the development of antibacterial agents (*1*).

UDP-*N*-acetylmuramoyl-pentapeptide is a precursor of peptidoglycan biosynthesis and is synthesized by a series of intracytoplasmic enzymes. Among these are MurC, MurD, MurE and MurF which add L-alanine, D-glutamic acid, L-lysine (or diaminopimelic acid), and D-alanyl–D-alanine dimers, respectively, to UDP-*N*-acetylmuramic acid (UDP-MurNAc) in an ATP-dependent manner (*2*, *3*). The primary sequences of MurC, MurD, MurE and MurF have conserved motifs in common with one another and thus, they appear to have evolved from a single progenitor enzyme (*4*). It has been suggested that the amino acid residues best conserved among these Mur ligases are critical for enzymatic function (*5*, *6*). Indeed, the catalytic steps carried out by each enzyme seem to be almost the same. In the case of MurC, the protein first binds to ATP and then can bind to UDP-MurNAc (*5*). Next, the carbonyl group of UDP-MurNAc is activated, forming an acyl phosphate intermediate *via* donation of the γ-phosphoryl group from ATP. Next, the nitrogen atom of the amino group of the ligand amino acid acts on the intermediate. This results in formation of a new peptide bond, together with the release of orthophosphate and ADP (*7*). Hydrolysis of ATP by MurC depends on both L-alanine and UDP-MurNAc. Structural information about Mur family proteins suggest that these ligases are topologically similar to one another and are each composed of three structural domains, an N-terminal UDP-MurNAc-binding domain, a central ATP-binding domain and C-terminal domain responsible for binding of substrate amino acids. The ATP-binding and ligand amino acid-binding domains of the four Mur proteins are also quite similar to one another from a

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structural viewpoint (*8*). A detailed structural analysis of *Escherichia coli* MurD revealed that a dynamic structural shift from an open structure to a closed structure is triggered by ATP binding (*9*).

MurC protein transfers the first amino acid of the pentapeptide. In an enzymological analysis of MurC with L-alanine analogues, MurC retained high substrate selectivity to the L-form (*7*). Analysis of the co-crystal structure of *Haemophilus influenzae* MurC with UDP-MurNAc-L-alanine and the non-hydrolyzable ATP analogue AMP-PNP (5′-adenylyl-β,γ-imidodiphosphate) suggested the presence of a shallow hydrophobic pocket that fit well with the short L-alanine side chain; moreover, the residues required for specific binding to L-alanine have been well conserved among MurC proteins but not in other Mur ligases (*10*, *11*), which is related to the fact that the concerned amino acids differ (D-glutamic acid, etc.). However, little validation *via* studying the effects of mutations on the structure of Mur ligases has been performed.

We have isolated temperature-sensitive mutations in the *Staphylococcus aureus murC* gene (*12*). Two of these have an amino acid substitution at His343, which corresponds to His376 of the *H. influenzae* MurC protein and forms the shallow hydrophobic pocket described above. Interestingly, the temperature-sensitive phenotype of the *murCH343Y* strains can be partially suppressed by multi-copy supply of the *aapA* gene, which encodes an alanine uptake transporter, and the suppression is allele specific, as the temperature-sensitive phenotypes of the *murCG222E* and *murCG222R* mutations were not suppressed under the same conditions. In addition, suppression of the temperature-sensitive phenotype of *murCH343Y* by multi-copy supply of the *aapA* gene was enhanced by addition of L-alanine to a high concentration in the medium (*12*). These results suggest that His343 of the *S. aureus* MurC is normally required for high-affinity binding of L-alanine to MurC at least at the restrictive temperature. However, this idea has not yet been tested directly *via* biochemical analyses.

In this work, we examine the role of *S. aureus* MurC His343 in L-alanine recognition, using both *in vitro* and *in vivo* assays, which is the first validation on the structure of MurC protein by studying the effects of a point mutation. We have also constructed a structural model of *S. aureus* MurC bound to both UDP-MurNAc-L-alanine and an ATP analogue, and discuss which amino acid residues appear to be required for L-alanine recognition. These results provide valuable insights that should prove useful for the development of antibiotics that target MurC proteins and Mur family ligases.

MATERIALS AND METHODS

Reagents—All reagents were of analytical grade. UDP-MurNAc was highly purified from a temperature-sensitive *S. aureus murC* mutant cells using HPLC (Yutsudo, T. *et al.*, unpublished data). The UDP-MurNAc was calculated according to the UV absorbance of UDP and the extinction coefficient for UDP was $\varepsilon_{262} = 10{,}000\ M^{-1}$ (*13*).

Strains and Growth Conditions—*Escherichia coli* strain JM109 or HB101 was used as a host for plasmid construction. The *S. aureus* temperature-sensitive *murC* mutants that grew at 30°C but not at 43°C, were derived from RN4220 *via* ethylmethane sulfonate mutagenesis (*12*). *Escherichia coli* and *S. aureus* cells were grown in LB medium containing 1% (w/v) Bacto tryptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl with 50 μg/ml ampicillin or 12.5 μg/ml chrolamphenicol as needed. Synthetic medium was prepared as described (*14*) with some modifications. The medium comprised 100 mM potassium phosphate pH 7.3, 10 μM calcium chloride, 50 μM iron (III) chloride, 500 μM magnesium sulphate, 1 μg/ml thiamine, 6 μg/ml nicotinic acid, 6.25 ng/ml biotin, 3.5 μg/ml calcium pantothenate, 2 μg/ml pyridoxal hydrochloride, 10 mM glucose and 17 amino acids (40 μg/ml of each) in which, L-alanine, L-serine and glycine were omitted. As indicated, synthetic medium supplemented with 100 μg/ml L-alanine, L-serine or glycine was also used.

Plasmids for Overexpression of S. aureus MurC—To construct plasmids for overexpression of *S. aureus* MurC in *E. coli*, the *S. aureus* MurC open reading frame was amplified by PCR using the primer 5′-AAGGAGTT TTATA**TCATGA**CACACTATCA-3′, which has a *Bsp*HI site (boldface type) and the initiation codon, and the primer 5′-TATAAGCTTATTAAAACGCATTTTTCATGC-3′. The p*murC* plasmid, which harbours wild-type *S. aureus murC* (*12*) or chromosomal DNA from the *murCH343Y* mutant strain TS9214 (*12*) was used as a template. Each PCR-amplified product was digested with *Bsp*HI and *Hin*dIII and then ligated into *Nco*I, *Hin*dIII-digested pET21d (Novagen), resulting in pET*murC* or pET*murCH343Y*.

Purification of S. aureus MurC—*Escherichia coli* BL21 (DE3)/pLysS cells (Novagen) were transformed with pET*murC*, and the resultant strain was grown in 1 l of LB medium at 37°C until the OD_{600} reached 0.5, at which point the concentration of IPTG in the medium was adjusted to 0.5 mM. After a 2 h induction period, cells were harvested by centrifugation, washed once with 30 ml of 0.9% (w/v) NaCl and frozen in liquid N_2. The frozen cell paste (~3 g) was re-suspended and lysed in 6 ml of lysis buffer [50 mM HEPES–KOH, pH 7.6, 1 mM DTT, 1 mM EDTA, 20% (v/v) glycerol, 0.25 M KCl, 0.3 mg/ml lysozyme (Sigma) and 20 mM spermidine-HCL (Sigma)] on ice for 30 min. Samples were then re-frozen, thawed and sonicated four times for 30 s using a Branson Sonifier 450 with an ultramicrotip. The resulting homogenate was centrifuged at 145,000*g* for 30 min at 4°C, and cleared lysates obtained (Fraction I) were adjusted to 0.4 g/ml ammonium sulphate and stirred for 20 min at 4°C. After centrifugation at 40,000*g* for 30 min, the precipitate was collected and dissolved in buffer C′ [50 mM HEPES–KOH, pH 7.6, 1 mM DTT, 1 mM EDTA and 20% (v/v) glycerol] and dialysed against the same buffer (Fraction II). Fraction II was loaded at a flow rate of 0.27 ml/min onto a DEAE-cellulose column (DE52, Whatman) that had been pre-equilibrated with buffer C′ and then eluted with a linear gradient from 0 to 0.8 M NaCl in buffer C′. Active wild-type MurC was identified in the 0.3 M NaCl eluate. The pooled MurC fraction

(Fraction III) was dialysed against buffer C′, loaded onto a MonoQ HR5/5 column (Amersham Biosciences) at a flow rate of 0.25 ml/min, and eluted with a linear gradient from 0 to 1 M NaCl in buffer C′. Active wild-type MurC eluted at 0.25 M NaCl was pooled (Fraction IV). MurC protein was followed by monitoring UDP-MurNAc:L-alanine ligase activity. MurCH343Y protein was purified in a similar way except that *E. coli* BL21 (DE3)/pLysS transformed with pET*murCH343Y* were used and cultured at 30°C. In each case, the purity of Fraction IV exceeded 80% as determined after separation by SDS–PAGE. The protein concentration was determined using the Bradford method (Bio-Rad) with BSA as a standard. Prior to use in other assays, the purified proteins were stored at −80°C.

MurC Ligase Assay—To measure the MurC activity in crude enzyme fractions during purification, the ligase activity was determined by monitoring incorporation of L-[^{14}C]alanine into UDP-MurNAc (*13*). A standard reaction volume of 12.5 μl was used and the reaction was performed in a solution of 100 mM Tris–HCl (pH 8.5), 25 mM $(NH_4)_2SO_4$, 20 mM $MgCl_2$, 2 mM β-mercaptoethanol, 100 μM L-[^{14}C]alanine, 2 mM ATP and 150 μM UDP-MurNAc. Reactions were carried out for 20 min at 37°C for wild-type MurC or 30°C for the MurCH343Y mutant protein. The reactions were stopped by addition of 5 μl of acetic acid; next, 3.5 μl of the resulting mixtures were spotted onto silica-gel TLC plates. The plates were developed with isobutylic acid:1 M NH_4OH (5:3), followed by exposure to an Imaging plate, BAS MS2025 (Fuji) and the plates were analysed with BAS-1800II (Fuji).

MurC ATPase—Catalytic constants of MurC proteins were measured by the ATP hydrolysis activity of MurC using purified enzyme fractions, which was determined by monitoring the release of phosphoric acid (*7*, *15*). A standard reaction volume of 12.5 μl was used and the reaction was performed in a solution of 100 mM Tris–HCl (pH 8.5), 25 mM $(NH_4)_2SO_4$, 20 mM $MgCl_2$, 2 mM β-mercaptoethanol, 10 mM L-alanine, 300 μM ATP and 300 μM UDP-MurNAc. Reactions were carried out for 20 min at 30°C for both wild-type and mutant enzymes and then, 0.1 ml of BIO MOL GREEN reagent (BIO MOL) were added. After 30 min at room temperature, the reactions were assayed for absorbance at 610 nm. Orthophosphate was used as a standard.

Molecular Modelling of the S. aureus MurC Protein—The *S. aureus* MurC–UDP-MurNAc-L-alanine–AMP-PNP complex was drawn based on the atomic coordinates for the crystal structure of the *H. influenzae* MurC–UDP-MurNAc-L-alanine–AMP-PNP complex being available in Protein Data Bank (http://www.rcsb.org/pdb/home/home.do) under accession # 1p3dA (*10*) and those for the *S. aureus* MurC structure model being available in ModBase (http://modbase.compbio.ucsf.edu/modbase-cgi/search_form.cgi) under accession # Q6G8I7 using Swiss-PdbViewer 3.6b2, which automatically fitted UDP-MurNAc-L-alanine and AMP-PNP from the *H. influenzae* MurC–UDP-MurNAc-L-alanine–AMP-PNP complex into the *S. aureus* MurC structure *via* 3D matching. The graphical representation was rendered using POV-Ray 3.6.

RESULTS AND DISCUSSION

Purification of Wild-Type and H343Y Mutant MurC Proteins—Towards the goal of determining if the *S. aureus murCH343Y* mutation reduces the affinity of MurC for L-alanine, wild-type and MurCH343Y MurC proteins were first purified. Overproduction of *S. aureus* MurC proteins was carried out in *E. coli* cells as described in MATERIALS AND METHODS section. In *E. coli* harbouring the wild-type or mutant constructs, a protein with a molecular mass of 49 kDa, which corresponds to the estimated molecular mass of *S. aureus* MurC protein, was induced in an IPTG-dependent manner. Because crude enzyme fractions should contain other ATPases with strong activity, we measured the amount of ligase activity that was quantified on TLC by determining the difference in mobility between L-[^{14}C]alanine and UDP-MurNAc-L-[^{14}C]alanine (Fig. 1A). We noted a soluble cell lysate fraction in which the UDP-MurNAc:L-alanine ligase activity was increased, consistent with the presence of the wild-type or mutant *S. aureus* MurC protein (Fig. 1B). Each of the recombinant *S. aureus* MurC proteins was precipitated with ammonium sulphate and separated using DEAE-cellulose and a MonoQ column (Table 1). In MonoQ column chromatography, the ligase activity of each protein eluted as a single peak. In addition, the ligase activity was co-eluted with the ATPase activity that depended on the presence of both UDP-MurNAc and L-alanine in each protein (data not shown). Moreover, an ~49 kDa protein was detected after SDS–PAGE and eluted with a similar profile to that observed for the two activities described above (data not shown). The salt concentration competent for elution of ligase activity was similar for the wild-type and mutant proteins. Recovery of the ligase activity into Fraction IV from Fraction I was 52 and 23% for wild-type and mutant MurC, respectively (Table 1), suggesting that major fraction of the ligase activity was effectively purified in either case. The purity of the Fraction IVs for wild-type or mutant MurC exceeded 80% as measured after separation by SDS–PAGE and visualization of proteins with CBB R-250 (data not shown). Additionally, the ATPase activity of either Fraction IV was dependent on UDP-MurNAc (Fig. 2), suggesting that contamination by other ATPases was negligible. Thus, to further analyse the recombinant wild-type or mutant MurC, we performed the assays using Fraction IV. The ligase activity of wild-type MurC was much the same as that observed for the native *S. aureus* MurC protein, which has been reported elsewhere (*16*).

For the MurCH343Y protein, the recovery of ligase activity was lower than the wild-type protein (Table 1), while we should mention that comparison of the activities between the enzymes shown in Table 1 was inappropriate because reaction conditions for mutant protein differed with that of wild-type protein on incubation temperature and L-alanine concentrations. When their ATPase activities were assayed in the same conditions, the H343Y protein recovered had a 3-fold lower specific activity than the wild-type protein (Fig. 2, Table 2). One interpretation of the results is that the purification step damages the mutant protein and

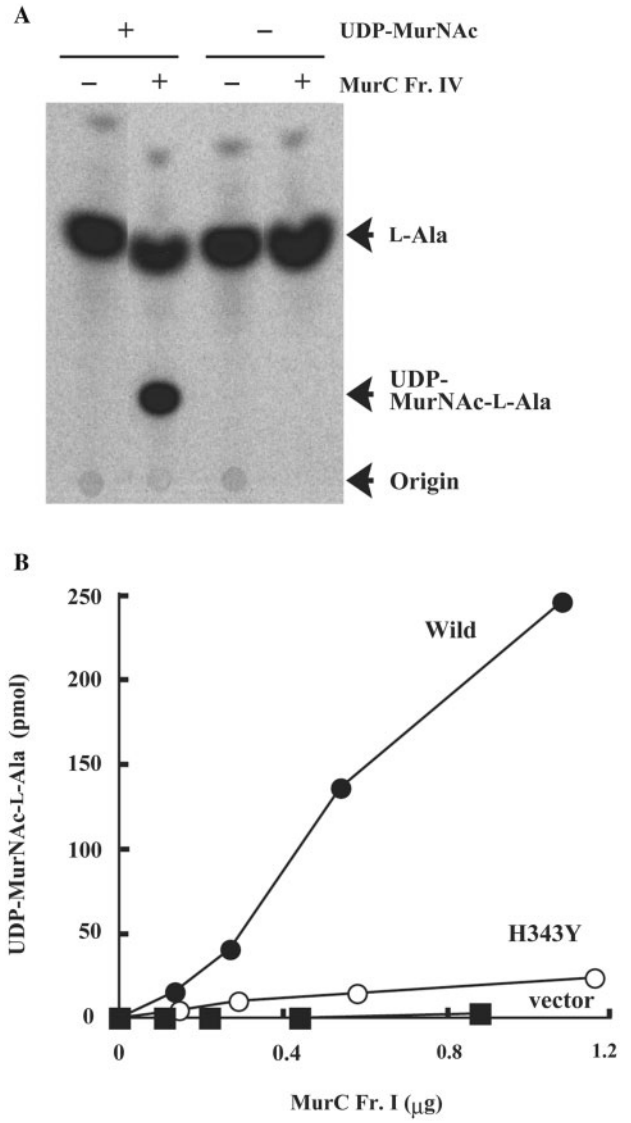

Fig. 1. **The UDP-MurNAc:L-alanine ligase activity of *S. aureus* MurC proteins.** (A) Determination of the UDP-MurNAc:L-alanine ligase activity on TLC. A wild-type MurC fraction, Fraction IV (800 ng), was incubated with 0.1 mM L-[^{14}C]alanine and 2 mM ATP in the presence or absence of 0.15 mM UDP-MurNAc. Samples were spotted onto silica-gel TLC plates and developed with isobutylic acid:1 M NH_4OH (5:3), and plates were analysed by fluorography. (B) Expression of *S. aureus* MurC ligases in *E. coli*. Fraction I was prepared from *E. coli* cells harbouring a plasmid for overexpression of *S. aureus* wild-type MurC (closed circle) or MurC H343Y (open circle), or an empty vector control (closed square) grown in the presence of IPTG. MurC ligase activity in either Fraction I was assayed at 30°C in a standard reaction mixture that included 0.1 mM L-[^{14}C]alanine, 2 mM ATP and 0.15 mM UDP-MurNAc.

purified mutant protein fraction contains some inactivated enzymes.

Characterization of the Wild-Type and Mutant Enzymes—The kinetic constants describing the effect of each substrate on ATPase activity of the purified recombinant wild-type and mutant MurC proteins were determined in the presence of moderate levels of the other substrates at the same conditions (Table 2). At 30°C, the K_m for L-alanine with MurCH343Y protein was 3.2 mM, whereas that with the wild-type protein was 0.44 mM, or a >7-fold difference (Fig. 3). In contrast, the K_m values for UDP-MurNAc and ATP were much the same with the purified mutant and wild-type proteins (Fig. 4), suggesting that the H343Y mutation has little effects on overall structure of MurC protein. In either case, we did not detect the presence of another enzyme containing different K_m values. The H343Y protein showed comparative ATPase activity at 43°C as wild-type protein when they were assayed in excess amount of L-alanine using equivalent units of activity those were determined at 30°C. This result was consistent with the facts that the temperature-sensitive cell growth of *murCH343Y* was suppressed by multi-copy supply of alanine transporter *aapA* gene, which was enhanced by a simultaneous addition of L-alanine to the medium (*12*). A larger K_m value was observed for L-alanine with MurCH343Y as compared with that of wild-type protein when the assays were performed at 43°C (data not shown). Taken together, these results suggest that the H343Y mutation selectively lowers the utilization of MurC protein for L-alanine. Specifically, the H343Y mutation might reduce the affinity of MurC protein for L-alanine and/or reduce the rate of following catalytic reaction including attack of the nitrogen atom of the amino group of L-alanine on the carbon atom of the terminal carbonyl group of the acyl phosphate intermediate.

Alanine Stimulates Growth of the H343Y Mutant Strain—As shown above, the biochemical analysis suggests that MurCH343Y has a reduced affinity for L-alanine at 30°C. Thus, it seems possible that the *murCH343Y* mutant requires a higher cellular concentration of L-alanine than parental strain for growth at 30°C. To examine this, we next determined the requirement for L-alanine of the temperature-sensitive *murCH343Y* mutant strain for growth at 30°C. In synthetic medium lacking L-alanine, L-serine and glycine, the *murCH343Y* mutant strains TS9214 and TS9224 (*12*) can grow but their rates of growth are slower than wild-type. In contrast, the growing ability of the *murCG222E* mutant strain TS2913 (*12*) was comparable to that of the parental strain (Fig. 5). The Gly222 residue is not a conserved residue in bacterial MurCs and is not involved in specific substrate binding, and the *murCG222E* is a temperature-sensitive mutation whose temperature-sensitive cell growth is not suppressed by the multi-copy supply of the *aapA* gene (*12*). This allele-specific slow growth phenotype of *murCH343Y* was suppressed by introduction of a plasmid with the wild-type *murC* gene, suggesting the phenotype was caused by the *murCH343Y* mutation (data not shown). In addition, the slow-growth phenotype could be suppressed by addition of L-alanine to the medium, but not by addition of serine or glycine (Fig. 5). Specifically, the addition of serine slowed the growth of not only the *murCG222E* mutant but also the *murCH343Y* mutants, the reduced MurC ligase activity in cells seemed to be a reason for the slower growth rate caused by the addition of serine. Then, addition of glycine increased the growth rate of not only

Table 1. **Purification table for wild-type and H343Y mutant *S. aureus* MurC proteins.**

Fraction	Purification step	Total protein (mg)	Specific activity[a] (U/mg)	Total activity (U)	Yield (%)	Purification (fold)
Wild-type						
I	Cell lysate	208	17	3,494	100	1
II	$(NH_4)_2SO_4$ ppt.	165	18	2,987	85	1.1
III	DEAE-cellulose	45	43	1,909	55	2.5
IV	MonoQ	17	110	1,848	52	6.4
H343Y						
I	Cell lysate	215	14	3,032	100	1
II	$(NH_4)_2SO_4$ ppt.	135	15	1,995	66	1.1
III	DEAE-cellulose	48	17	806	27	1.2
IV	MonoQ	23	30	693	23	2.1

[a]1 U defined 1 nmol L-alanine incorporation to UDP-MurNAc/min under standard ligase assay condition except for using 0.1 mM or 1 mM L-[^{14}C]alanine for wild-type or H343Y protein, respectively. Reactions were at 37°C or 30°C for wild-type protein or H343Y protein, respectively.

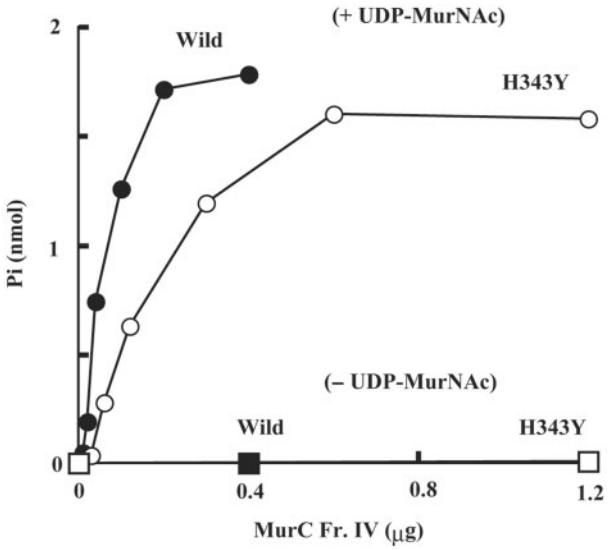

Fig. 2. **ATPase activity of MurC proteins.** MurC ATPase activity was determined using different amounts of purified (Fraction IV) wild-type MurC (closed symbol) or H343Y mutant MurC protein (open symbol) in the presence (circle) or absence (square) of UDP-MurNAc at 30°C in a standard ATPase reaction mixture that included 10 mM L-alanine, 0.3 mM ATP and 0.3 mM UDP-MurNAc.

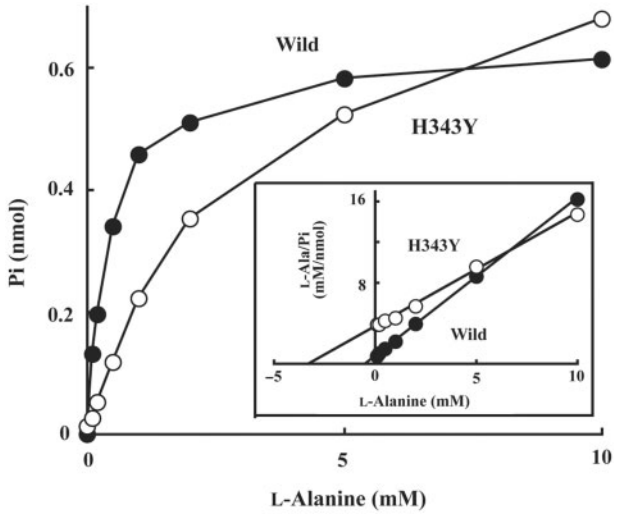

Fig. 3. **L-Alanine-dependent ATPase activity of MurC proteins.** MurC ATPase activity was determined using different concentrations of L-alanine in the presence of 100 ng of wild-type MurC (closed circle) or 300 ng of the H343Y mutant MurC protein (open circle) in a standard ATPase reaction mixture that included 0.3 mM ATP and 0.3 mM UDP-MurNAc. Insert, $[S]_0/v \sim [S]_0$ plot.

Table 2. **Kinetic parameters of *S. aureus* MurC proteins.**

Parameter[a]	Wild-type	H343Y
V_{max} (nmol/mg of protein/min)	660	200
K_m, L-alanine (mM)	0.44	3.2
K_m, UDP-MurNAc (mM)	0.24	0.21
K_m, ATP (mM)	0.10	0.14
V_{max}/K_m^{Ala} (nmol/mg of protein/min/mM)	1,500 (100)	65 (4.3)
V_{max}/K_m^{UM} (nmol/mg of protein/min/mM)	2,800 (100)	990 (35)
V_{max}/K_m^{ATP} (nmol/mg of protein/min/mM)	6,600 (100)	1,500 (23)

[a]Data from Figs 2–4, in which reactions were carried out at 30°C for both wild-type and mutant enzymes in a standard ATPase reaction mixture, were summarized. The numbers in parentheses are relative values, the enzymatic activity of the wild-type protein being considered as 100%. UM:UDP-MurNAc.

the *murC* mutants but also parent strain, and importantly, the growth rate of the *murCH343Y* mutants were still slower than the parent strain, suggesting the slow growth phenotype of the *murCH343Y* mutants were not suppressed by the addition of glycine (Fig. 5). As the K_m value for L-alanine with MurCH343Y (3.2 mM; Table 2) is much higher than the K_m for free L-alanine with a number of other enzymes [*i.e.* 10–150 μM for bacterial MurC (*5*, *7*, *13*, *15*, *17–19*), 0.97 mM for *E. coli* alanine racemase (*20*), and 0.24 mM for L-alanine tRNA synthetase (*21*)] it seems reasonable to propose that the high K_m causes the allele-specific slow-growth phenotype on alanine-depleted synthetic medium. Moreover, our results are consistent with previous observations for MurCR327A from *E. coli*, in which *E. coli* MurCR327A resulted in an increase in the K_m for L-alanine to 12.6 mM *in vitro* but had similar K_m values for UDP-MurNAc and ATP, in addition to a loss of its ability to complement temperature-sensitive cell growth of an

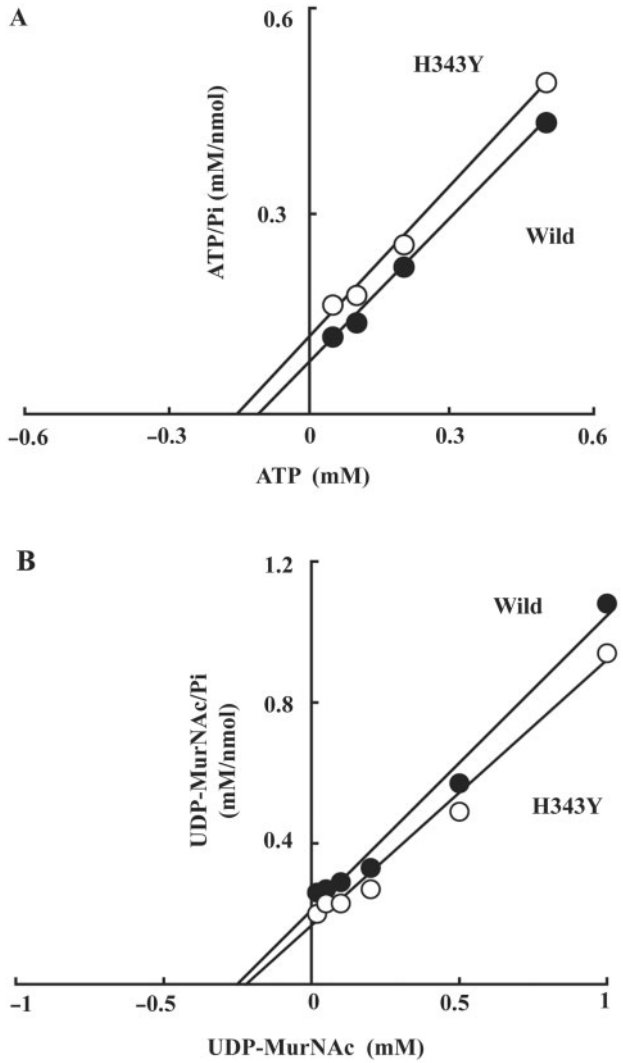

Fig. 4. **ATP or UDP-MurNAc-dependent ATPase activity of MurC proteins.** (A) MurC ATPase activity was determined using different concentrations of ATP in the presence of 100 ng wild-type MurC (closed circle) or 300 ng of the MurC H343Y protein (open circle). (B) MurC ATPase activity was determined using different concentrations of UDP-MurNAc in the presence of 100 ng of wild-type MurC (closed circle) or 300 ng of MurC H343Y protein (open circle). Each was in a standard ATPase reaction condition that included 10 mM L-alanine and either 0.3 mM UDP-MurNAc in (A) or 0.3 mM ATP in (B).

E. coli murC mutant (*5*). Taken together, these results suggest that an increase in the concentration of intracellular alanine, resulting from uptake of alanine from the medium and *de novo* synthesis of alanine, plays a stimulatory role in cell growth of the *murCH343Y* mutant cells. Thus, consistent with the results of the biochemical analysis, His343 appears to be critical *in vivo* for high-affinity binding of L-alanine by the MurC protein at 30°C.

Homology Modelling of S. aureus MurC Protein—To help elucidate the roles of His343 and other residues that may be involved in L-alanine recognition in *S. aureus* MurC, a structural model of *S. aureus* MurC protein bound to ligands was created, based on the co-crystal structure of *H. influenzae* MurC and its ligands (*10*). The structural model of *S. aureus* MurC revealed that His343 forms a shallow hydrophobic pocket that, together with Tyr313, His315 and Ala417, supports the short side chain of L-alanine (Fig. 6). The model also revealed that His343, together with Arg347, is adjacent to the carbonyl group of UDP-MurNAc-L-alanine, as both residues are part of the β18–α12 loop (Fig. 6). This result is consistent with the results of structural analysis of other MurCs (*10*, *11*), MurD (*22*) and MurE (*23*). That is, for each of these, charged residues have been conserved among the ligases present in the β18–α12 loops of other MurCs (*e.g.* Arg380 of *H. influenzae* MurC), in the corresponding β17–α13 loop of *E. coli* MurD (Lys348), or in the corresponding β18–α13 loop of *E. coli* MurE (Arg389), and in each case, the residues are located near the carbonyl group of the ligand amino acid. Extending this further, it seems reasonable to predict that of the candidate residues of *E. coli* MurF (namely, Asp360, Glu363, Leu364 and Gly365), it is either the charged residue Asp360 or Glu363, both of which have been well conserved, that is present in the corresponding β19–α13 loop and functions in substrate D-alanyl-D-alanine recognition.

Interestingly, Ala417 on the β21–α15 loop of *S. aureus* MurC has been conserved among MurC proteins but not Mur ligases. As for residues on the β18–α12 loop of MurC (see above), the β21–α15 loop of MurC, the corresponding β20–α16 loop of MurD and the corresponding β21–β22 loop of MurE have residues conserved among each ligase in the group but not among all Mur ligases. These residues have in common that they are involved in recognition of substrate amino acid side chains.

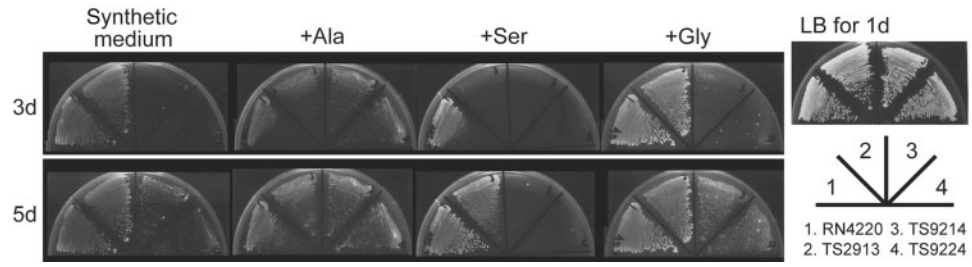

Fig. 5. **Effect of alanine on growth of *murCH343Y* mutant cells.** An overnight culture of the bacterial strains was diluted to OD_{600} 0.01 in synthetic medium and then streaked on synthetic medium agar plates with or without L-alanine, L-serine or glycine, or on LB agar plates. The plates were incubated at 30°C for the indicated number of days. RN4220, parental strain; TS2913, *murCG222E*; TS9214 and TS9224, *murCH343Y*.

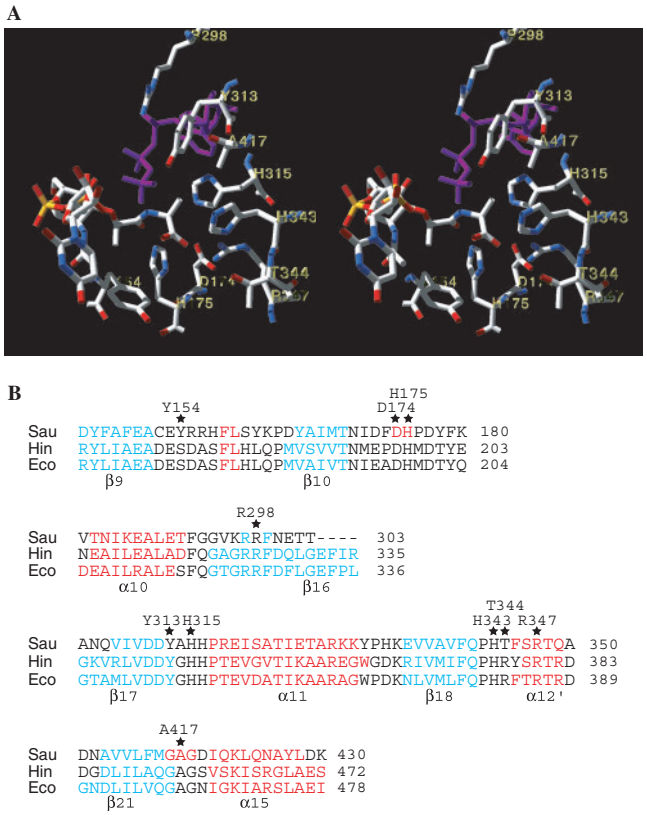

Fig. 6. **Amino acid residues in *S. aureus* MurC required for L-alanine recognition.** (A) Residues within 5 Å from either carbon, nitrogen or oxygen atom of the alanyl group of UDP-MurNAc-L-alanine or Arg298, mapped on a *S. aureus* MurC homology model (Q6G817), which was superimposed using the Swiss-Pdb viewer program on the structure of UDP-MurNAc-L-alanine and AMP-PNP (purple) obtained from the *H. influenzae* MurC–UDP-MurNAc-L-alanine–AMP-PNP complex structure (1p3dA). (B) Multiple alignments with secondary structure information were shown for MurC protein from *Staphylococcus aureus* (Sau, Q6G817), *Haemophilus influenzae* (Hin, 1p3dA), *Escherichia coli* (Eco, 2F00). Residues that consist of β-sheet or α-helix are in blue or red, respectively. Numbering of either β-sheet or α-helix is from Smith, C.A. 2006. Gaps (-) were introduced to optimize the alignment. Asterisks above the alignment indicate the residues mapped in (A).

Specifically, Ser415 and Phe422 in *E. coli* MurD and Gly464 and Glu468 in *E. coli* MurE play roles in the enzyme-substrate side chain interaction (*22*, *23*). Thus, the functions of the loop structures, *i.e.* recognition of specific substrate amino acids, have been conserved among the various sub-groups of Mur ligases.

Tyr313 and His315 in the shallow hydrophobic pocket are located on the β17–α11 loop of *S. aureus* MurC (Fig. 6). Characteristically, Asp312 on the same loop interacts with the ribose moiety of AMP-PNP (data not shown). The observation that the residue on the β17–α11 loop that interacts with ADP ribose is adjoined to the residue responsible for recognition of the substrate alanine seems to be important. That is, the linking of these two may help to bring the ligand close to the catalytic site and thus, contribute to the efficiency with which the catalytic reaction occurs. A similar structural property was observed for the *E. coli* MurD β16–α12 loop (*22*). In *E. coli* MurE, the hydrophobic region of the substrate diaminopimeric acid lay against the ring of His359 on the β17–α12 loop. However, the role of the *E. coli* MurE Asp356 for ribose recognition has not been fully revealed, at least in part because there has not yet been a report of the co-crystal structure of MurE with an ATP analogue (*23*). The differences in the residues that participate in substrate amino acid recognition among Mur ligases seem to achieve selective binding to different ligand amino acids.

Residues close to the alanine of UDP-MurNAc-L-alanine in the *S. aureus* MurC include Tyr154, Asp174, His175 and Thr344 (Fig. 6). Among these, Asp174 and His175 (which correspond to Asp198 and His199 of *E. coli* MurC) have been well conserved among bacterial MurC proteins and His175 among all Mur ligases. Involvement of the residue corresponding to *S. aureus* His175 in the active site has also been elucidated by mutational analysis (*5*, *6*) and these results were consistent with the structural model of *S. aureus* MurC presented here (Fig. 6).

The present results indicate the specific involvement of *S. aureus* MurC His343 in L-alanine recognition, adding to what was previously known for Arg327 of *E. coli* (*5*), which is similarly involved in substrate alanine recognition but presumably serves a different specific role. An argnine in the position occupied by *E. coli* Arg327 has been well conserved among Mur ligases. Structural modelling of *S. aureus* MurC has showed that the corresponding Arg298 accesses the ligand L-alanine from a different angle from that observed for His343 and further, that the Arg residue interacts with the α-phosphate of ATP (Fig. 6)(*10*, *11*). The corresponding Arg302 of *E. coli* MurD has also been shown to interact with the α-phosphate of ATP (*22*). Based on the structure, one would predict that the arginine is involved in ATP binding; however, biochemical analysis of *E. coli* MurCR327A revealed its specific importance for utilization of alanine (*5*). Interestingly, a considerable shift in the position of *E. coli* MurD Arg302 upon binding to ADP has been described (*22*). Thus, the corresponding arginine in each Mur ligase might change position dramatically and in an enzyme status-dependent manner, thus playing a role in affinity of the ligand amino acid or in helping to attack the nitrogen atom of the amino group of the ligand amino acid on the carbon atom of the terminal carbonyl group of the acyl phosphate intermediate. Alternatively, *E. coli* MurCR327A protein may have an altered structural conformation when involved in L-alanine recognition. The latter explanation seems consistent with the *S. aureus* MurC model, in which the corresponding Arg298 is located far from the alanyl group of UDP-MurNAc-L-alanine (Fig. 6).

Conclusion—H343Y has been identified as a temperature-sensitive mutation of *S. aureus* MurC. The wild-type residue is predicted to form the shallow hydrophobic pocket that catches the short side chain of the MurC substrate, L-alanine. Temperature-sensitive cell growth of the *murCH343Y* mutant strain can be partially suppressed by multi-copy supply of the *aapA* gene,

which encodes a transporter of alanine, glycine and serine. In this study, we examined the role of His343 for alanine recognition both *in vitro* and *in vivo*. We found that the H343Y mutation increased the K_m value for L-alanine >7-fold (from 0.44 to 3.2 mM) but did not have a large effect on the K_m values for UDP-MurNAc and ATP. The *murCH343Y* mutant strain has an allele-specific slow-growth phenotype at permissive temperature in medium deficient for alanine that can be suppressed by addition of L-alanine. In a structural model of *S. aureus* MurC, His343 appears to form a shallow hydrophobic pocket and is adjacent to the carboxyl group of the alanine in UDP-MurNAc-L-alanine. Thus, according to the results of both *in vitro* and *in vivo* analyses, His343 appears to be important for recognition by MurC protein of L-alanine specifically. Furthermore, the results indicate that structures of *E. coli* and *H. influenzae* MurC proteins (*10*, *11*) are in good agreement with the enzymatic characteristics of MurC as observed in *in vitro* and *in vivo* studies.

The results presented here should help to make it possible to design inhibitors that specifically target residues involved in binding of ligand amino acids.

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