Addictive plasmids drive hospital transmission of mupirocin-resistant *Staphylococcus aureus*

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Key words: Mupirocin-resistance, MRSA, transmission, stringent response

SUMMARY (297 of 300 words)

Background

Mupirocin, a widely used topical agent for decolonization of *Staphylococcus aureus*, is increasingly compromised by resistance. Although plasmid-mediated mupirocin resistance is a recognized cause of decolonization failure, its role in facilitating hospital-wide transmission is unknown.

Methods

We conducted genomic surveillance of *S. aureus* at two interconnected urban hospitals where mupirocin decolonization is routine. Genome sequencing of >10,000 isolates was integrated with patient data to identify transmission and resistance determinants. Bacterial phenotypes and fitness were evaluated *in vitro* and in murine colonization models.

Findings

Genome sequencing identified 475 hospital transmission events; none were detected by conventional surveillance. The *mupA* (*ileS2*) resistance determinant, carried on conjugative plasmids, was enriched eightfold in methicillin-resistant *S. aureus* (MRSA) relative to methicillin-susceptible strains. *mupA* was associated with nearly a threefold greater chance of hospital transmission, especially within endemic healthcare-associated MRSA lineages, and was enriched twofold in hospital-onset infections compared with admission colonizing isolates. Multiple independently evolved inactivating mutations in the essential chromosomal gene *ileS1* co-occurred with *mupA*, creating plasmid addiction in which *mupA* became indispensable for bacterial survival. Addiction arose most frequently within the dominant community-acquired MRSA lineage, where plasmid carriage reduced colonization fitness in mice. Plasmid-containing strains exhibited stringent-response activation, explaining the fitness costs and collateral tolerance to disinfectants, such as ethanol and peroxide. Although addiction reduced *S. aureus* fitness, it increased plasmid transfer, and addicted variants spread across hosts, demonstrating adaptation that mitigates these costs. Unexpectedly, we identified a mupirocin–dependent vulnerability to isoleucine limitation, revealing a potential strategy to target *mupA*-mediated resistance.

Interpretation

Plasmids promote hospital transmission of mupirocin-resistant *S. aureus* and create an evolutionary trap in which antibiotic use selects for bacterial dependence on otherwise costly

resistance elements. This dependence revealed a collateral bacterial vulnerability that could be exploited to target resistant strains and preserve the effectiveness of mupirocin.

Funding

National Institutes of Health.

Research in context (394 words)

Evidence before this study

We searched PubMed for articles published in any language from database inception to July 2025 using the terms "*Staphylococcus aureus*," "MRSA," "mupirocin," "chlorhexidine," "resistance," "plasmid," "addiction," and "transmission." We also reviewed the reference lists of relevant studies. Previous work showed that mupirocin resistance, mediated either by non-inactivating chromosomal *ileS1* mutations or plasmid-encoded *mupA* genes, decreases the success of *S. aureus* decolonization efforts. However, no study had systematically examined how plasmid-mediated mupirocin resistance affects *S. aureus* transmission within hospitals. Existing literature describes fitness costs of mupirocin plasmids, but not mechanisms that enforce plasmid maintenance through gene essentiality. Additionally, the relationship between mupirocin resistance, stringent response activation**,** cross-tolerance to other disinfectants, and collateral vulnerabilities has not been reported.

Added value of this study

This study provides the first comprehensive genomic evidence that plasmid-mediated mupirocin resistance directly contributes to *S. aureus* transmission in hospitals. By sequencing thousands of isolates from two interconnected hospitals, we show that plasmids encoding *mupA* (*ileS2*) are strongly associated (11·3% increase in nosocomial transmission, 95% CI 5.8–16·9) with nosocomial spread. We further identify a previously undescribed form of plasmid addiction caused by inactivation of the essential chromosomal gene *ileS1*, rendering plasmid-encoded *ileS2* indispensable for survival. Addiction did not itself enhance strain fitness but stabilizes otherwise costly resistance elements, enabling their continued transmission and dissemination. By revealing an unexpected dependence of MRSA on a resistance plasmid, our findings identified a collateral vulnerability to isoleucine limitation that could be leveraged to sustain the effectiveness of mupirocin.

Implications of all available evidence

Our findings highlight a crucial paradox: mupirocin decolonization works—susceptible strains transmit less—but its use selects for a previously unappreciated form of plasmid-addicted strain having cross-tolerance to multiple disinfectants. Addiction helps explain the maintenance of resistance plasmids that drive MRSA spread within hospitals. At the same time, resistance-fitness interactions that create genetic dependencies also expose collateral vulnerabilities, providing a rationale for resistance-breaking adjuvant strategies aimed at preserving the effectiveness of mupirocin. By showing how antimicrobial use can create irreversible genomic dependencies, this study also reframes infection-control strategies toward proactive genomic surveillance to identify and mitigate the unintended consequences of mupirocin use. More broadly, the work challenges the assumption that reducing antibiotic exposure alone will reverse resistance once genetic dependence has evolved.

INTRODUCTION (3,498 of 3,500 words)

Nasal mupirocin, often combined with chlorhexidine bathing, is widely used for decolonization and control of *Staphylococcus aureus*, especially methicillin-resistant strains (MRSA).[1,2] These interventions help sustain recent reductions in healthcare-associated infections.[3] Although mupirocin use selects for resistance,[4] its effects on hospital-wide transmission and clonal dynamics are unknown. As the principal decolonizing agent worldwide, the emergence and spread of resistance threaten infection prevention in vulnerable patients.

Mupirocin resistance arises through two mechanisms. Low-level resistance results from point mutations in the essential chromosomal isoleucyl-tRNA synthetase gene *ileS1*, whereas high-level resistance is mediated by *mupA*, encoding an alternative isoleucyl-tRNA synthetase (*ileS2*),[5] carried on plasmids.[6] Plasmid-mediated resistance is enriched in settings having extensive topical antimicrobial use. We recently reported the emergence of a dual mupirocin-resistant community-acquired (CA-)MRSA lineage undergoing community transmission in New York City.[4] However, whether such plasmids promote hospital transmission and what evolutionary forces maintain[7] them is unknown.

Here, we analyzed >10,000 *S. aureus* genomes collected between January 2022 and January 2025 to define the distribution, spread, and evolutionary consequences of *mupA*-carrying plasmids. Strong selection for *mupA* was evident in preferential nosocomial transmission of *mupA*-carrying strains, and more strikingly, in the repeated emergence (>100 independent times) of inactivating *ileS1* mutations that created plasmid addiction. In these strains, plasmid-encoded *ileS2* becomes essential for survival. The association of addiction with increased conjugative transfer of *mupA* and broad biocide tolerance suggests additional selective pressures reinforcing plasmid maintenance and spread. Conversely, mupirocin unexpectedly synergized with exogenous isoleucine to re-sensitize *mupA*-positive strains.These findings underscore the need for genomic surveillance and resistance testing. They also suggest that risk-targeted decolonization strategies and exploitation of plasmid-dependent vulnerabilities may help control mupirocin-resistant *S. aureus*.

METHODS

Methods are summarized here; additional details are provided in the Supplementary Materials.

Study isolates and procedures

S. aureus isolates were obtained from an institutional biobank at two tertiary-care hospitals within NYU Langone Health (Brooklyn and Manhattan; 444 and 813 beds). The biobank and decolonization procedures have been described previously.[8,9] For the present study, we analyzed an expanded surveillance cohort comprising 10,321 sequenced isolates collected between January 2022 and January 2025, including 4,129 MRSA and 6,192 MSSA isolates. MRSA isolates were obtained from various clinical and admission screening cultures, whereas MSSA isolates were obtained from bloodstream infection and admission screening cultures only. Adult patients admitted to medicine, oncology, transplant, and intensive care units underwent routine admission screening for nasal colonization. Patients colonized with MRSA or MSSA received twice-daily intranasal mupirocin and daily bathing with 2% chlorhexidine gluconate for 5 days. During the study period, admission screening compliance was 77.8%. Among colonized patients, 49% received at least one day of decolonization therapy and 10.8% received three or more days.

To identify putative progenitors of the *ileS1* mutant lineage E499, we screened a historical collection of 1,649 consecutive clinical MRSA isolates collected between 2017 and 2020 (pre-COVID-19 pandemic). These 604 isolates were obtained from bloodstream, respiratory, and wound infections in adult patients. The 31 *mupA*-positive CC8 isolates with closed genomes analyzed for chromosomal integration were also derived from this historical collection.

The study was approved by the NYU Langone Health Institutional Review Board (s24-01872). Additional details regarding strains, plasmids, primers, growth conditions, RNA sequencing, and phenotypic assays are provided in the Supplementary Methods.

Genome sequencing and transmission detection

Whole-genome sequencing, assembly, quality control, bioinformatic analyses, and genomic surveillance–based transmission inference were performed as previously described for the institutional genomic surveillance program.[8,9] Additional details, including transmission cluster definitions and linkage criteria, are provided in the Supplementary Methods.

Role of the funding source

The study sponsors had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all study data and had final responsibility for the decision to submit for publication.

RESULTS

Genomic Epidemiology of *mupA*-positive *S. aureus*

Susceptibility testing for mupirocin resistance is not routinely performed by most clinical laboratories because resistance is not included on automated testing platforms. We therefore used whole-genome sequencing to systematically identify *mupA* across 10,321 *S. aureus* isolates collected through institutional genomic surveillance. Overall, *mupA* was detected in 11·8% of isolates, indicating a substantial reservoir of mupirocin resistance within the healthcare system.

Mupirocin resistance was strongly enriched in MRSA. Although MSSA isolates were more numerous overall (6,192 versus 4,129), 85·8% (1,041) of *mupA*-positive isolates occurred in MRSA. Phylogenetic analysis revealed, as previously reported,[10] that *mupA* within MRSA was concentrated in CC8 and, to a lesser extent, CC5. These lineages represent the predominant community- and healthcare-associated MRSA lineages, respectively, in New York City and the United States.[9] Together, these lineages accounted for 96·7% of *mupA*-positive MRSA, whereas the remainder of *mupA*-positive isolates occurred in sporadic isolates and small clusters. We therefore focused subsequent analyses on CC8 and CC5.

mupA was distributed across multiple independent branches of both the CC5 and CC8 phylogenies (Fig. 1A), consistent with repeated horizontal acquisition, not single ancestral expansion. Nevertheless, several large clonal expansions were evident, particularly within CC8.

Assembly of a subset of complete plasmids revealed several structurally distinct plasmid groups (representative plasmid in Fig. 1B; group comparisons in Supplementary Fig. 1). These complete plasmids informed plasmid classification across the surveillance cohort (Fig. 1). Three major plasmid groups (Groups 1–3) accounted for >91% of *mupA*-positive isolates, with Group 1 representing 60%. Thus, *mupA* dissemination was dominated by a small number of plasmid groups. Mobility predictions of the reference plasmids corresponded closely with the major structural groups and were consistent with a recent regional survey (Supplementary Fig. 1);[11] representative Group 1 plasmids were conjugative, Group 2 included mobilizable and non-mobilizable plasmids, and Group 3 plasmids were mobilizable. Conjugative plasmids contained a *tra* region homologous to those of the *S. aureus* resistance plasmids pUSA03 and pSK41.[6,12]

Plasmids exhibited extensive mosaicism, consistent with recombination and exchange of accessory genetic elements (Supplementary Fig. 1). In addition to *mupA*, these plasmids encoded multiple accessory features, including antimicrobial resistance determinants, transposases, and insertion sequences. Notably, 53·7% of *mupA*-positive strains also carried chlorhexidine resistance genes, establishing dual resistance to both hospital decolonization agents, consistent with previous reports.[4] Some plasmids also carried a pentose phosphate pathway locus arranged in a non-native, operon-like configuration (Fig. 1B).

Mapping plasmid structural groups onto the chromosomal phylogeny revealed both multiple plasmid groups within individual lineages and identical plasmid groups across lineages (Fig. 1A). Thus, *mupA* plasmids disseminate through a combination of vertical clonal expansion and repeated inter-lineage transfer.

mupA is Associated with Increased Patient-to-patient Transmission

Patients harboring *mupA*-positive isolates differed in several previously identified risk factors for *S. aureus* colonization and transmission, including greater hospital exposure (Supplementary Table 1).[9] To determine whether *mupA* was associated with transmission, we analyzed transmission using a previously described genomic-epidemiologic framework (Supplemental Methods).[8,9] Compared with *mupA*-negative strains, *mupA*-positive strains had a 10·5 percentage point higher transmission rate (95% CI 8·4 – 12·7); this association was robust to propensity score adjustment for lineage, hospitalization history, antibiotic exposure, and demographic factors (adjusted difference 11·3 percentage points, 95% CI 5·8–16·9; Supplementary Table 2). Consistent with transmission contributing to infection risk,[13] *mupA* was also enriched among hospital-onset infections compared with admission colonizing isolates (17·0% vs 10·2%).

The transmission advantage associated with *mupA* was observed across both MRSA and MSSA but was most pronounced in MRSA lineages (Fig. 2, Supplementary Table 3). MSSA increased from 2·1% to 7·0% with *mupA* but remained low overall; mupirocin-resistant MSSA transmitted at rates comparable to mupirocin-susceptible MRSA. In CC5 MRSA, transmission increased from 12·9% to 26%, and in CC8 MRSA the increase was from 7·4% to 14·6%. Thus, although *mupA* was most prevalent in CC8, its association with transmission was greatest in CC5, the predominant healthcare-associated MRSA lineage.

Chromosomal *ileS1* point mutations conferring low-level mupirocin resistance (V588F, V631F, G593V)[14] were not associated with increased transmission. The consistency of the *mupA* effect across genetic backgrounds and patient populations, its persistence after statistical adjustment, and the absence of effect from chromosomal resistance mutations support a role for plasmid-mediated mupirocin resistance in promoting hospital transmission.

Recurrent Evolution of Plasmid Addiction Through Chromosomal *ileS1* Inactivation

Unexpectedly, analysis of *ileS1* revealed widespread inactivation of this essential chromosomal isoleucyl-tRNA synthetase among *mupA*-positive isolates. We identified 105 distinct loss-of-function mutations, consisting of frameshifts and truncations (Fig. 3A). Most mutations were observed in only one or a few isolates; however, several underwent substantial clonal expansion, including one lineage involving >155 patients. Isolates carrying the same inactivating *ileS1* mutation clustered within monophyletic groups, indicating that recurrently observed mutations reflected clonal expansion rather than parallel evolution (Fig. 3B; Supplementary Fig. 2).

Addiction (*leS1* inactivation–mediated dependence on *mupA*) was most common in CC8 and in plasmid groups associated with this lineage, reflecting the high prevalence of *mupA* and expansion of addicted clones. Thus, plasmid addiction both reflects MRSA population structure and contributes to its evolution.

Because chromosomal integration events are largely invisible in short-read surveillance data, we screened all available closed CC8 genomes from our collection. Among 31 *mupA*-positive isolates, chromosomal integration was identified in 6 (19·4%). All six occurred among the seven addicted isolates (85·7%), involving three independent addiction variants, whereas none of the 24 non-addicted isolates showed chromosomal integration. Thus, chromosomal acquisition of *mupA* may be an additional outcome of plasmid addiction.

Fitness Costs of *mupA* Plasmids Drive Selection for Addiction

To determine whether *ileS1* inactivation conferred a fitness advantage, we introduced an *ileS1* addiction mutation identified in a clinical isolate into a laboratory strain carrying a *mupA* plasmid. Addiction increased neither resistance nor growth in the presence of subinhibitory mupirocin concentration (Supplementary Fig. 3). Instead, addicted strains grew more poorly than their plasmid-carrying parental counterparts, indicating that addiction imposes a burden rather than enhancing resistance.

These findings suggest addiction is selected not to improve bacterial growth but to stabilize plasmids that would otherwise be lost. Consistent with this interpretation, plasmid carriage produced no measurable defect *in vitro* across a range of growth conditions, but it imposed substantial costs *in vivo*. In a murine nasal colonization and transmission model (Fig. 4A), introduction of the plasmid into a CC8 background, where addiction is common, significantly reduced both colonization and transmission (Fig 4B, Supplementary Fig. 4A). By contrast, in CC5, where addiction is rare, plasmid carriage had minimal or slightly beneficial effects (Fig. 4C, Supplementary Fig. 4B). Introduction of an addicted chromosomal background further reduced transmission (Supplementary Fig. 5), demonstrating that addiction can persist despite exacerbating plasmid-associated fitness costs. These *in vitro* and *in vivo* observations suggest that *mupA* plasmids impose lineage-specific fitness costs and suggest that such costs help drive both selection for addiction and its uneven distribution across MRSA populations.

***mupA* Plasmids/Addiction Impose Dependence on the Stringent Response**

Because aminoacyl-tRNA synthetases operate near saturation, even small activity reductions impose steep fitness costs.[15] Reduced charging efficiency of the *mupA*-encoded isoleucyl-tRNA synthetase may therefore elevate uncharged tRNA levels and activate the stringent response via production of (p)ppGpp, a nutrient-stress pathway triggered by accumulation of uncharged tRNA that suppresses growth and biosynthesis.[16] Experimental disruption of the stringent-response is complicated in *S. aureus* because the RelA/SpoT homolog (RSH), which mediates (p)ppGpp synthesis and hydrolysis, is essential for viability.[17] Therefore, we evaluated growth in the presence of relacin, an inhibitor of (p)ppGpp synthesis, to test whether *mupA*-carrying strains exhibit increased dependence on stringent-response signaling, as predicted for aminoacyl-tRNA synthetase limitation.[15] Relacin impaired growth of plasmid-carrying and addicted strains, but it had little effect on plasmid-free, parental strains (Fig. 5). Although relacin exhibits low affinity for Rel and may have off-target effects,[18] these findings support the idea that

mupA plasmids increase dependence on the stringent response. This dependence provides a mechanistic explanation for both the fitness cost of plasmid carriage and selection of addicted *ileS1* mutants.

Because stringent-response activation promotes biofilm formation,[19] we examined this phenotype. Addicted strains, but not their plasmid-carrying parental counterparts, exhibited increased biofilm formation compared with plasmid-free controls (Fig. 6A). Because conjugative transfer is enhanced within biofilms,[20] we next tested whether addiction influences plasmid transfer. Conjugation frequencies were higher in addicted strains than in their plasmid-carrying parental strains (8·88e-7 vs 2·31e-7 (mean values); Fig. 6B). Thus, addiction not only ensures vertical inheritance but it also promotes horizontal dissemination, thereby expanding the reservoir of transmissible resistance.[21]

Stringent-Response Dependence Creates a Therapeutic Vulnerability

Expression analysis of selected stringent-response genes in biofilm populations was consistent with activation of stringent-response pathways in both *mupA* plasmid-carrying and addicted strains (Supplementary Fig. 6), as evidenced by increased expression of oligopeptide transport genes (*oppA*, *oppC*), branched-chain amino acid (BCAA) biosynthesis genes (*ilvB*, *leuA*, *leuB*), purine salvage (*xpt*), and reduced expression of ribosomal genes (*rplR*, *rplV*), purine biosynthesis (*purH)*, and metabolism (*sucA*, *atpA, gltB*).[22] The addicted strain generally showed larger effect sizes across several markers, potentially explaining its association with increased biofilm formation. No change in *rsh* expression was observed, consistent with prior work showing that stringent-response transcriptional programs can persist after adaptation to sublethal stress without increased *rsh* transcription.[23] Reduced translational capacity and impaired energy metabolism provide a potential mechanistic basis for the fitness cost of plasmid carriage.

The stringent-response dependence imposed by *mupA*, and more generally by mupirocin, suggested a vulnerability arising from the interaction between stringent-response signaling and CodY-mediated regulation. In *S. aureus*, intracellular pools of BCAA are integrated with stringent-response signaling through the global regulator CodY.[24,25] When BCAA are abundant, CodY represses genes involved in amino acid biosynthesis and stress adaptation; depletion of these metabolites relieves repression and activates these pathways. Thus, exogenous isoleucine can uncouple this regulatory relationship by reactivating CodY even when stringent-

response signaling persists.[25] Among BCAA, isoleucine is the most potent mediator of this effect, restoring CodY-dependent repression despite continued metabolic stress.[25] This creates a regulatory mismatch in which stringent-response signaling remains active while CodY suppresses the biosynthetic programs needed for growth.

This interaction has limited consequences under nutrient-replete laboratory conditions, where amino acids are abundant. However, the *in vivo* environment differs substantially. Nasal secretions, the primary ecological niche for *S. aureus* colonization, are markedly deficient in free amino acids, especially isoleucine.[26] Under these conditions, mupirocin stringent-response activation occurs in the setting of amino-acid limitation, where relief of CodY repression would be expected to facilitate biosynthesis and growth. Exogenous isoleucine disrupts this adaptive response by restoring CodY despite ongoing stringent-response signaling, with consequences for growth.[25]

Consistent with these observations, moderate concentrations of isoleucine alone modestly impaired growth with both plasmid-free and plasmid-carrying strains grown in defined medium lacking BCAA (Fig. 7A). However, when combined with subinhibitory mupirocin, which further impairs isoleucyl-tRNA charging and amplifies stringent-response signaling, even lower doses of exogenous isoleucine produced near-complete growth collapse (Fig. 7B). Under these conditions, either agent alone had no impact. This synergistic interaction demonstrates a vulnerability in *mupA*-containing strains during mupirocin exposure, allowing resistance to be sensitized without directly inhibiting the resistance determinant.

Identifying vulnerabilities is important because inhibition of tRNA synthetases is known to induce broad tolerance to disinfectants.[27] The resulting stringent response suppresses the reactive oxygen species (ROS) surge induced by bactericidal agents, thereby reducing killing and promoting survival under biocidal stress. We therefore tested whether *mupA* plasmids altered tolerance to common biocidal agents after growth in BCAA-deficient medium, conditions that approximate the amino-acid limitation of nasal secretions. Plasmid-carrying strains exhibited a 1–2 log reduction in killing following exposure to multiple disinfectants, including ethanol, 2-propanol, and hydrogen peroxide, versus a plasmid-free parental strain (Fig. 8). In contrast, addicted strains did not show further increases in tolerance beyond that conferred by the plasmid alone. Thus, *mupA* plasmids confer broad biocide tolerance.

Discussion

The present work shows that *mupA* can lock *S. aureus* into plasmid carriage, stabilizing mupirocin resistance. In these cases, mutational inactivation of the essential chromosomal *ileS1* gene renders bacterial survival dependent on plasmid-encoded *ileS2*, creating plasmid-host interdependence that ensures retention even in the absence of mupirocin selection. This mechanism follows the logic of toxin-antitoxin systems—plasmid loss is more costly than retention—but loss achieves dependence through essential-gene replacement rather than post-segregational killing.[28] Addiction arose repeatedly, underscoring the likelihood of strong selection to stabilize resistance plasmids. Dissemination of addicted lineages beyond hospitals would entrench resistance and pose a public-health threat.

Chromosomal integration of *mupA* among addicted strains is consistent with the expectation that beneficial accessory genes may ultimately be captured by the chromosome.[29] However, unlike classical models, addiction creates dependence on a plasmid-borne function through loss of the chromosomal homolog. Thus, addiction may represent another route by which plasmid-borne genes become assimilated into the host genome.

Addiction increased biofilm formation and conjugative transfer of *mupA*. Thus, addiction not only seems to ensure vertical inheritance but also promotes horizontal dissemination, generating a plasmid reservoir that spreads resistance within and across MRSA lineages. Consistent with these experimental findings, phylogenetic analyses revealed expansion of addicted clones and repeated acquisition of *mupA*-containing plasmids (Figure 2A, Supplementary Fig. 2). Together, these findings indicate addiction promotes both persistence and spread of *mupA*.

Notably, *mupA* itself was associated with an increased likelihood of transmission, consistent with an advantage that was strongest in MRSA and greatest within CC5, the predominant healthcare-associated MRSA lineage, whereas the smallest effect was observed in MSSA. Thus, *mupA* preferentially enhanced transmission among strains that already exhibit the highest baseline transmission potential in healthcare settings (Figure 2, ref.[9]). Rather than creating successful lineages, *mupA* appears to amplify the fitness of strains already adapted to transmission. The molecular basis of this effect is unknown.

The observation that the absolute transmission advantage associated with *mupA* was greatest in healthcare-associated CC5 MRSA, despite the substantially higher prevalence of *mupA* in

CC8, suggests that healthcare transmission is only one component of the selective environment shaping *mupA* dissemination. Consistent with this idea, our prior work identified the emergence of a mupirocin-resistant CC8 lineage undergoing community transmission.[4] Additionally, the present study found that CC8, but not CC5, incurred a substantial *in vivo* fitness cost associated with *mupA* carriage and exhibited a markedly higher burden of addiction, suggesting stronger selection for plasmid stabilization in this lineage. Collectively, these findings, together with evidence that that transmission risk reflects interactions between host epidemiology and bacterial lineage,[9] suggest that distinct combinations of clonal spread, horizontal plasmid transfer, and plasmid addiction sustain *mupA* persistence in CC5 and CC8, thereby facilitating dissemination in both hospitals and communities even when selective pressure is reduced. Thus, strategies based solely on restricting mupirocin use may be insufficient.

mupA plasmids conferred increased survival under biocide pressure, including ethanol, isopropanol, and chlorhexidine. Increased tolerance to these agents could enhance environmental persistence and opportunities for transmission, potentially contributing to the increased transmission of *mupA*-carrying strains observed in our surveillance data. Together with additional metabolic and resistance determinants encoded by *mupA* plasmids, these findings suggest that the success of *mupA* plasmids may reflect broader adaptive benefits beyond resistance to mupirocin alone.

Exogenous isoleucine synergized with mupirocin to inhibit growth of *mupA*-containing strains under BCAA-limited, nasal-like conditions. A likely explanation (Supplementary Fig. 7) is that by impairing isoleucyl-tRNA charging, mupirocin reduces utilization of intracellular isoleucine, thereby increasing its effective availability. As a result, even low concentrations of exogenous isoleucine may be sufficient to maintain CodY-mediated repression of amino acid biosynthetic pathways. The resulting regulatory mismatch suppresses biosynthetic programs required for BCAA synthesis, creating a conditional vulnerability that culminates in growth collapse.

Further genomic and experimental studies are needed to confirm the predicted low charging efficiency of *mupA*-encoded IleS2, define how stringent-response activation generates plasmid-associated fitness costs, determine why these costs are particularly pronounced in CC8, and identify compensatory mutations that mitigate conflicts between plasmid carriage and the host chromosome in successful addicted lineages. Additional *in vivo* studies, including colonization and transmission models, are underway to determine whether the isoleucine-mediated

sensitization observed *in vitro* can be translated into an effective decolonization strategy. Finally, our epidemiologic analyses were limited to two hospitals within a single urban healthcare system and did not comprehensively sample community reservoirs. Because community-associated selection may contribute to *mupA* dissemination, studies spanning healthcare and community settings will be needed to define the prevalence and spread of addicted lineages.

Overall, our results suggest that reducing mupirocin use alone will be insufficient to reverse resistance, especially if addiction stabilizes resistance within anchor lineages: adaptive optimization may convert a costly plasmid into a self-sustaining component of the MRSA population. As with penicillin resistance, *mupA* could become endemic in hospitals and communities. This possibility underscores the need for new decolonization strategies that are less susceptible to resistance, including bacteriophage-derived lysins,[30] exploitation of collateral susceptibilities (e.g., isoleucine), and colonization-specific immunotherapy. However, development of new interventions alone may be insufficient. Preserving effectiveness of agents will require more judicious use, guided by identification of and targeted intervention in patients most likely to transmit or acquire MRSA rather than broad empiric treatment. Such risk stratification depends on genomic surveillance to identify transmission events, including those involving asymptomatic colonization, thereby defining high-risk spreaders and enabling development of personalized models that predict transmission. Because resistance may arise despite these efforts, surveillance will also be essential to detect emerging resistant clones before they adapt, spread, and become entrenched.[4] Prior work demonstrates that this is possible, but only real-time implementation will enable intervention before resistant lineages become endemic.[9]

Author Contributions:

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A.R.W., Investigation, Writing - review and editing
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O.O., Conceptualization, Data curation, Writing - review and editing
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Declaration of interests

B.S. has served on a scientific advisory board for Innoviva Specialty Therapeutics and has received research funding from Analog Devices Inc.

Data availability

Sequencing data are available through the NCBI repository using the accession number PRJNA1442056.

Acknowledgments

We thank Drs. Emily Grasso, Paul Zappile, Gael Wesby and Peter Meyn for assistance with library construction, and sequencing, Marc Lipsitch, Lorna Thorpe, and Jeffrey Weiser and for

critical comments on the manuscript, and Daria Frolova and John A. Lees for helpful discussions. This work was supported in part by National Institutes of Health grants AI137336 (to B.S. and A.R.); AI140754 (to B.S.); CDC U01CK000590 (B.S.), K08AI163457 (to R.J.U.), and funds from the NYULH Antimicrobial-Resistant Pathogens Program (B.S., S.H.). Genome sequencing was partially supported by Cancer Center Support Grant P30CA016087 from the NYULH Laura and Isaac Perlmutter Cancer Center.

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Figure legends

Figure 1. Distribution and characteristics of *mupA*-containing plasmids among epidemic MRSA lineages. (A) Maximum-likelihood phylogeny of CC5 and CC8 MRSA isolates from the surveillance cohort (*n* = 1,945), midpoint rooted. Clonal complexes are indicated on the tree. For each patient, a single representative susceptible or resistant isolate was included (<10 patients contributed both a *mupA*-positive and *mupA*-negative isolate). Outer ring, presence of *mupA* (red) or absence of *mupA* (gray). Middle ring, MOB-suite–imputed plasmid groups. Inner ring, major epidemic lineages (CC5, USA100 and USA800; CC8, USA300 and USA500), assigned by *in silico* detection of lineage-defining genetic elements and color-coded as indicated. Lineages were defined by the presence of characteristic genetic markers in >70% of isolates within each clonal complex, as described.[9] **(B)** Representative *mupA*-containing conjugative plasmid pMP1 (73,142 bp), illustrating Group 1, one of the three major *mupA*-associated plasmid groups identified in the surveillance cohort. pMP1 carries mupirocin resistance determinant *mupA* together with multiple insertion sequences and transposons, additional antimicrobial resistance determinants including the antiseptic resistance gene *qacC*, a phosphoenolpyruvate phosphatase (PPP) shunt metabolic operon, and genes involved in plasmid conjugation and mobilization. Representative plasmids from the three major plasmid groups and their structural relationships are shown in Supplementary Fig. 1.

Figure 2. *mupA* carriage is associated with increased transmission across diverse *S. aureus* lineages. (A) Transmission rates among *mupA*-positive (red) and *mupA*-negative (gray) isolates stratified by MSSA, MRSA CC5, MRSA CC8, and other MRSA clonal complexes. Bars indicate the proportion of isolates within each category linked to at least one transmission event. Transmission events were defined by combined genomic and epidemiologic linkage, including direct and indirect epidemiologic connections between patients carrying isolates separated by ≤20 single-nucleotide variants (Methods). In multivariable analyses, *mupA* carriage remained independently associated with transmission after adjustment for host and epidemiologic factors (Supplementary Table 2). **(B)** Schematic summary of the absolute increase in transmission associated with *mupA* carriage across lineage groups with differing baseline transmission frequencies. Values shown are derived from panel A and are presented to conceptually illustrate the relationship between baseline transmission and the magnitude of the *mupA*-associated transmission advantage. Numerical values, confidence intervals, and statistical comparisons are provided in Supplementary Table 3.

Figure 3. Diversity and phylogenetic distribution of chromosomal *ileS1* inactivation mutations among *mupA*-containing epidemic MRSA lineages. (A) Summary of insertion and deletion mutations predicted to inactivate chromosomal *ileS1* among *mupA*-positive MRSA isolates (*n* = 103). Each point represents a unique mutation identified among patient isolates, plotted according to its position within *ileS1*. Mutations are classified as frameshift (blue) or truncating (gold) variants. Amino acid positions are labeled using single-letter amino acid abbreviations. One representative isolate per patient was included in the analysis. The E499 truncation was the most frequently observed mutation. **(B)** Maximum-likelihood phylogeny of *mupA*-positive CC5 and CC8 MRSA isolates from the surveillance cohort (*n* = 574), midpoint rooted. Isolates correspond to the *mupA*-positive subset shown in Fig. 1A and are limited to a single representative isolate per patient. Clonal complexes, lineage assignments, and plasmid groups are annotated as described in Fig. 1. The outer ring indicates chromosomal *ileS1* inactivation mutations (frameshift or truncation). The largest cluster of *ileS1* mutants carries the E499 truncation and is further characterized in Supplementary Fig. 2.

Figure 4. Effect of the *mupA* plasmid on colonization and transmission in a neonatal murine model. (A) Experimental schematic. Neonatal mice (4–7 days of age) were intranasally inoculated with 10^6 CFU of the indicated *S. aureus* strain. Inoculated index pups were returned to their litters and cohoused with uninoculated littermates to assess transmission. Colonization was monitored daily by gently tapping the nares of each pup onto selective agar plates. At experimental endpoints, pups and dams were euthanized and upper respiratory tract colonization was quantified by retrograde nasal lavage and CFU enumeration. **(B)** Colonization and transmission of the CC8 strain LAC ± pMP1 (BS1158 and BS1963, respectively). **(C)** Colonization and transmission of the CC5 strain JH1 ± pMP1 (BS1231 and BS2045, respectively). Left, cumulative nasal colonization burden among inoculated index pups measured over days 1–7 post-inoculation. Middle, cumulative transmission burden among uninoculated contact pups measured over the same interval. Right, terminal upper respiratory tract colonization quantified by retrograde nasal lavage at the experimental endpoint. In **panel B**, green indicates the parental strain and red the *mupA*-plasmid–containing strain. In **panel C**, blue indicates the parental strain and red the *mupA*-plasmid–containing strain. Each symbol represents an individual animal. For longitudinal colonization measurements (left and middle panels), each symbol represents the cumulative CFU recovered from a single animal over the 7-day observation period. Whenever no *S.aureus* was recovered a value of 1 CFU was assigned. Horizontal bars indicate medians. All Y axes are in log scale. Daily colonization and

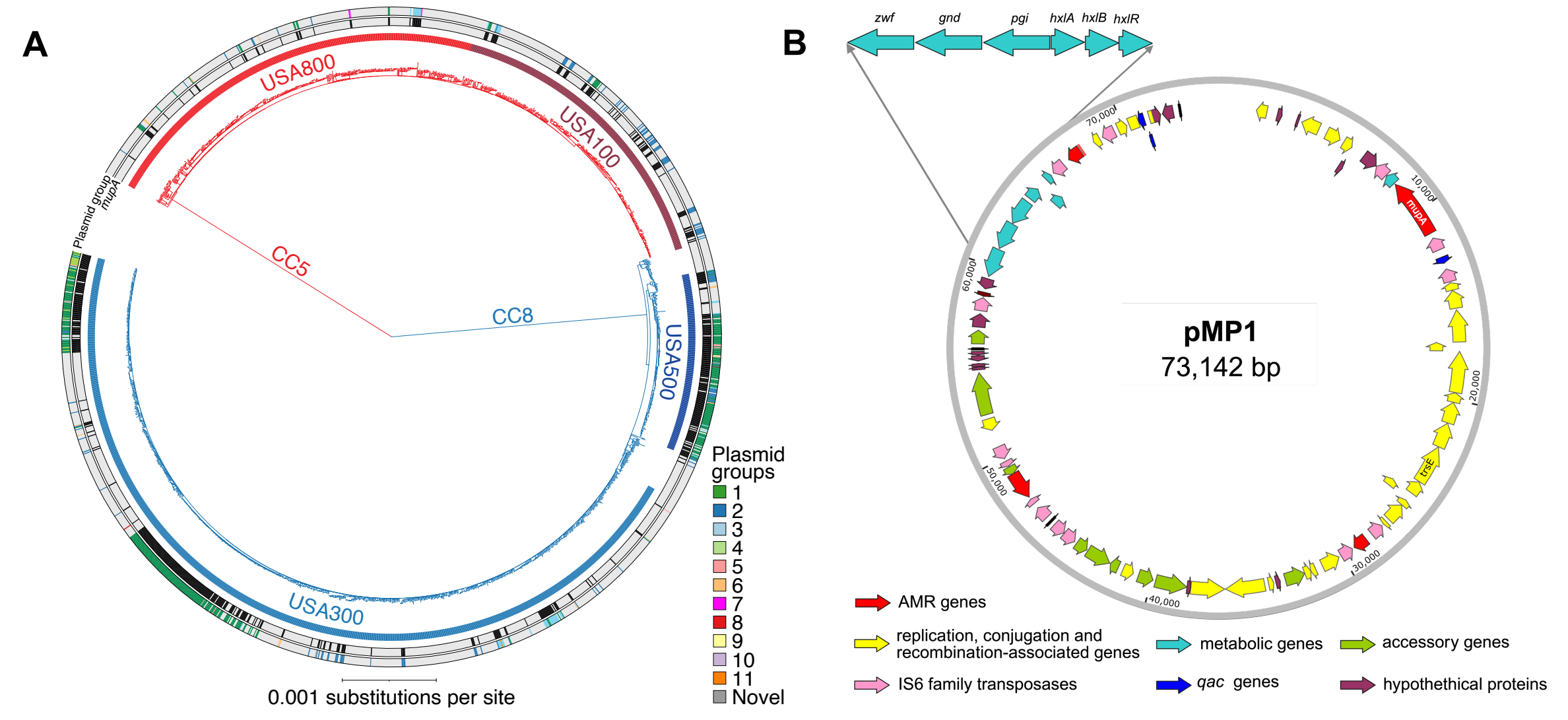
transmission kinetics for the experiments shown in panels B and C are presented in Supplementary Fig. 4.

Figure 5. Effect of Relacin on growth of *mupA*-containing strains with and without chromosomal *ileS1* inactivation. (A) Growth of *S. aureus* LAC wild-type (WT; BS1158), the isogenic *mupA* plasmid-containing derivative (pMP1; BS1963), and the addicted pMP1 *ileS1* Q299* mutant (BS2039) in TSB. Optical density at 600 nm wavelength was recorded using a BioTek LogPhase 600 microbiology reader. **(B)** Growth of the same strains in TSB as in A, supplemented with Relacin (at 8 mM). Data represent means ± SD from biological replicates (*n* = 3).

Figure 6. Effect of the *mupA* plasmid and chromosomal *ileS1* inactivation on biofilm formation and plasmid transfer. (A) Biofilm production *S. aureus* LAC wild-type (WT; BS1158), the isogenic *mupA* plasmid-containing derivative (pMP1; BS1963), and the addicted pMP1 *ileS1* Q299* mutant (BS2039). Biofilm biomass was quantified by crystal violet staining following 24 h of static growth and measured at OD_{600}. Bars represent mean ± SD. Data are from 4 independent experiments. Statistical significance was determined by one-way ANOVA with Dunnett's multiple-comparisons test; ****P ≤ 0.0001; ns, not significant. (**B**) Conjugative transfer of pMP1 by filter mating. Transfer frequencies were determined using either the parental LAC recipient strain (blue) or an isogenic pMP1-containing *ileS1* Q299* recipient strain (red). Each point represents an independent experiment (*n* = 5); lines connect paired replicate experiments. Conjugation frequencies are expressed as transconjugants per recipient. Statistical significance was determined by unpaired Student's t test; **P ≤ 0.01.

Figure 7. Exogenous isoleucine synergizes with subinhibitory mupirocin to impair growth of *mupA*-containing strains. (A) Growth of *S. aureus* LAC wild-type (WT; BS1158) and the isogenic *mupA* plasmid-containing derivative (pMP1; BS1963) in TSB medium, with or without supplementation with isoleucine at the indicated concentration. **(B)** Growth of the pMP1 strain in BCAA-free RPMI medium in which growth depends on endogenous BCAA biosynthesis, supplemented with subinhibitory mupirocin (100 µg/mL) alone or in combination with isoleucine at the indicated concentration. Growth was recorded using a BioTek LogPhase 600 microbiology reader, measuring OD_{600} at 40-min intervals. Data represent means ± SD from biological replicates (*n* = 3).

Figure 8. *mupA* plasmid increases broad-spectrum survival during disinfectant-mediated bacterial killing. Kinetics of killing by ethanol, 2-propanol and H_2O_2. Overnight cultures of *S. aureus* LAC (WT; BS1158) and the isogenic *mupA* plasmid-containing derivative (pMP1; BS1963) grown in BCAA- free RPMI medium, in which growth depends on endogenous BCAA biosynthesis, were diluted ($OD_{600} \approx 0.05$) into fresh tryptic soy broth (TSB) and grown with orbital shaking (180 rpm) to early exponential phase ($OD_{600} \approx 0.15$). Cultures were then exposed to **(A)** ethyl alcohol (20%), **(B)** 2-propanol (13%), or **(C)** hydrogen peroxide (H_2O_2; 20 mM) for the indicated times. Survival was quantified by CFU enumeration and expressed as $\log_{10}$ percent survival relative to CFU at time of disinfectant addition. Data represent means ± SD from biological replicates ($n = 3$). No differences in MICs for ethanol, 2-propanol, or H_2O_2 were detected between the parental and *mupA* plasmid-containing strains.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USA800
USA100
USA500
USA300
CC5
CC8
Plasmid group
mupA
0.001 substitutions per site
Plasmid groups
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2
3
4
5
6
7
8
9
10
11
Novel
B
zwf
gnd
pgi
hxlA
hxlB
hxlR
pMP1
73,142 bp
mupA
trsE
10,000
20,000
30,000
40,000
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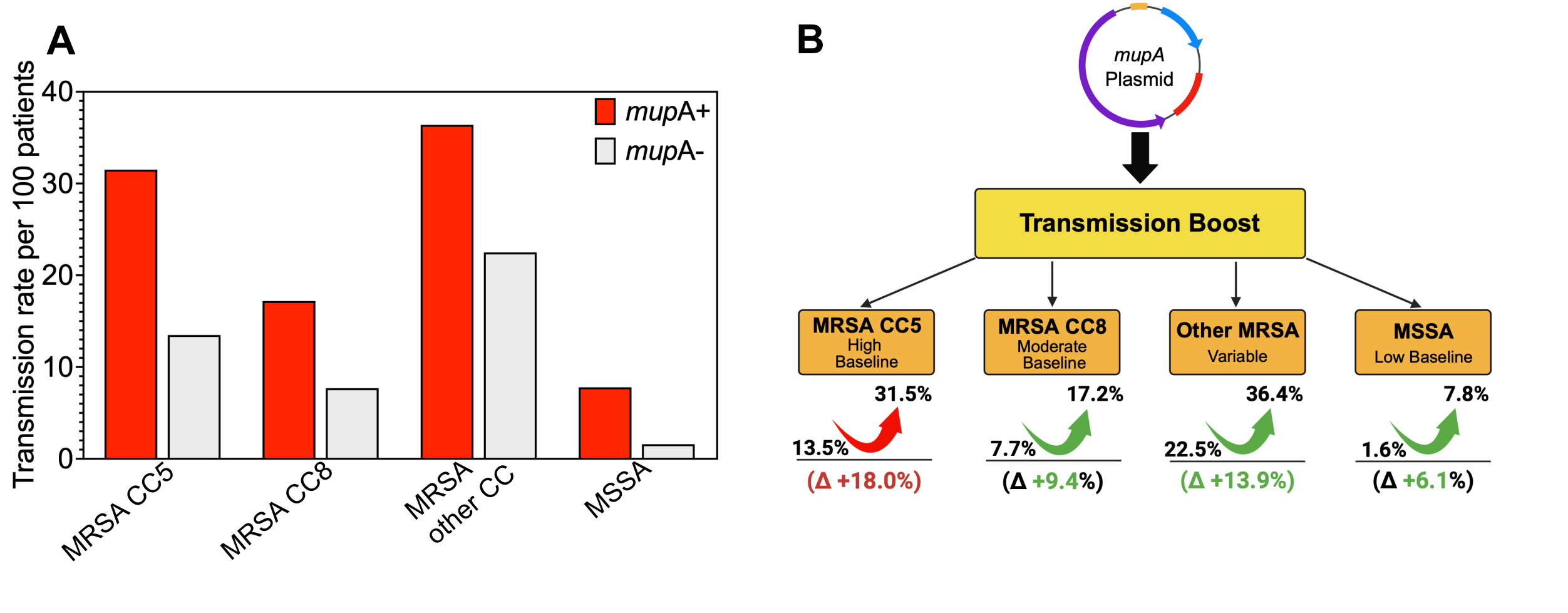
qac genes
accessory genes
hypothetical proteins


A
Transmission rate per 100 patients
40
30
20
10
0
mupA+
mupA-
MRSA CC5
MRSA CC8
MRSA other CC
MSSA
B
mupA Plasmid
Transmission Boost
MRSA CC5
High Baseline
MRSA CC8
Moderate Baseline
Other MRSA
Variable
MSSA
Low Baseline
31.5%
13.5%
(Δ +18.0%)
17.2%
7.7%
(Δ +9.4%)
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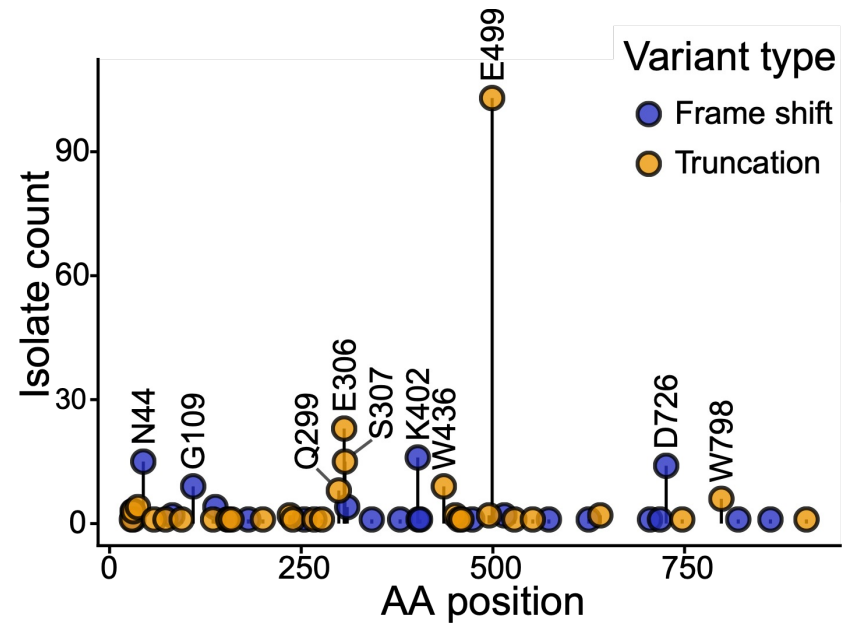
(Δ +13.9%)
7.8%
1.6%
(Δ +6.1%)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Variant type
Frame shift
Truncation
Isolate count
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60
30
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0
250
500
750
AA position
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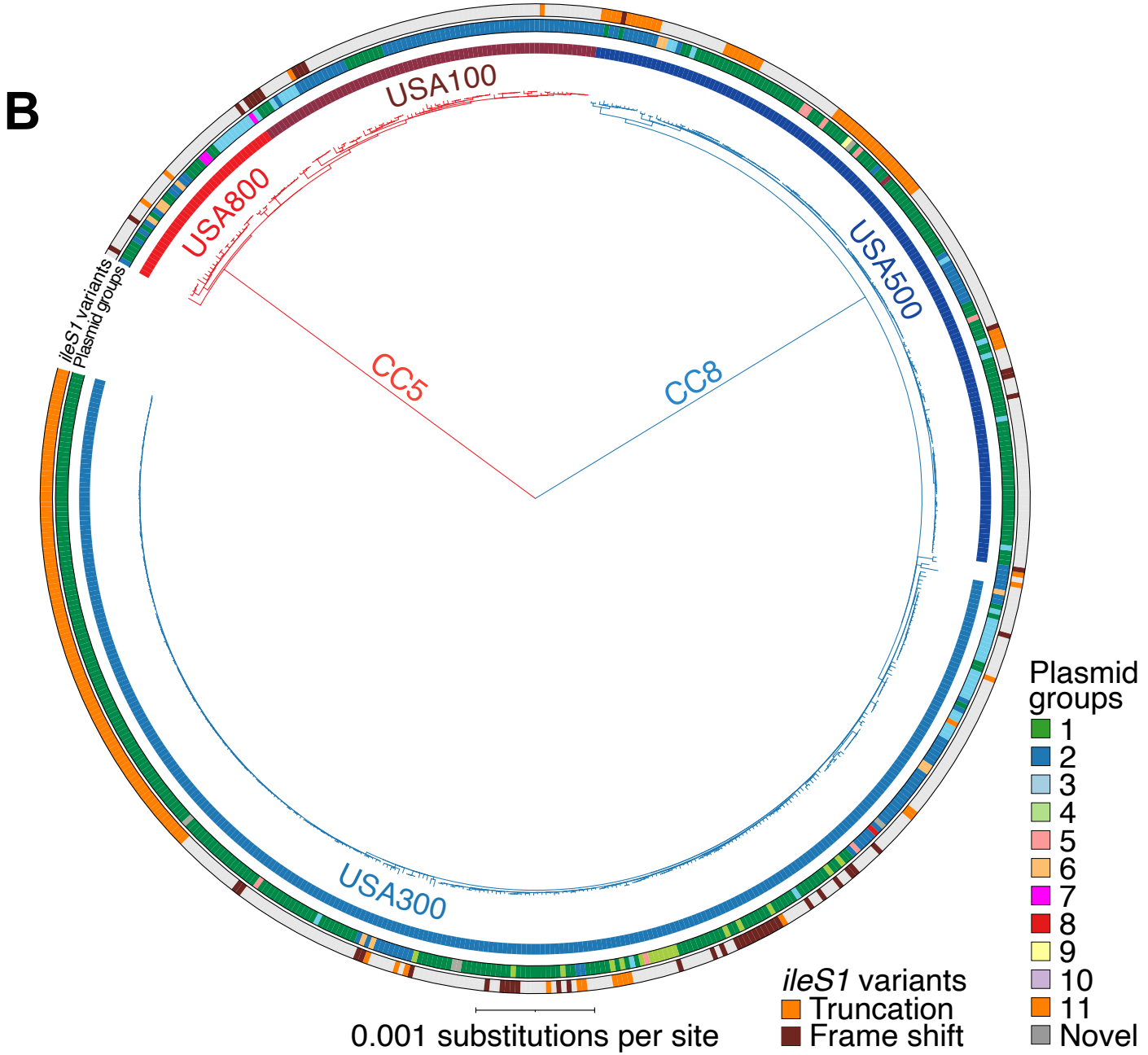
G109
Q299
E306
S307
K402
W436
E499
D726
W798


B
USA100
USA800
USA500
CC5
CC8
USA300
ileS1 variants
Plasmid groups
0.001 substitutions per site
Plasmid groups
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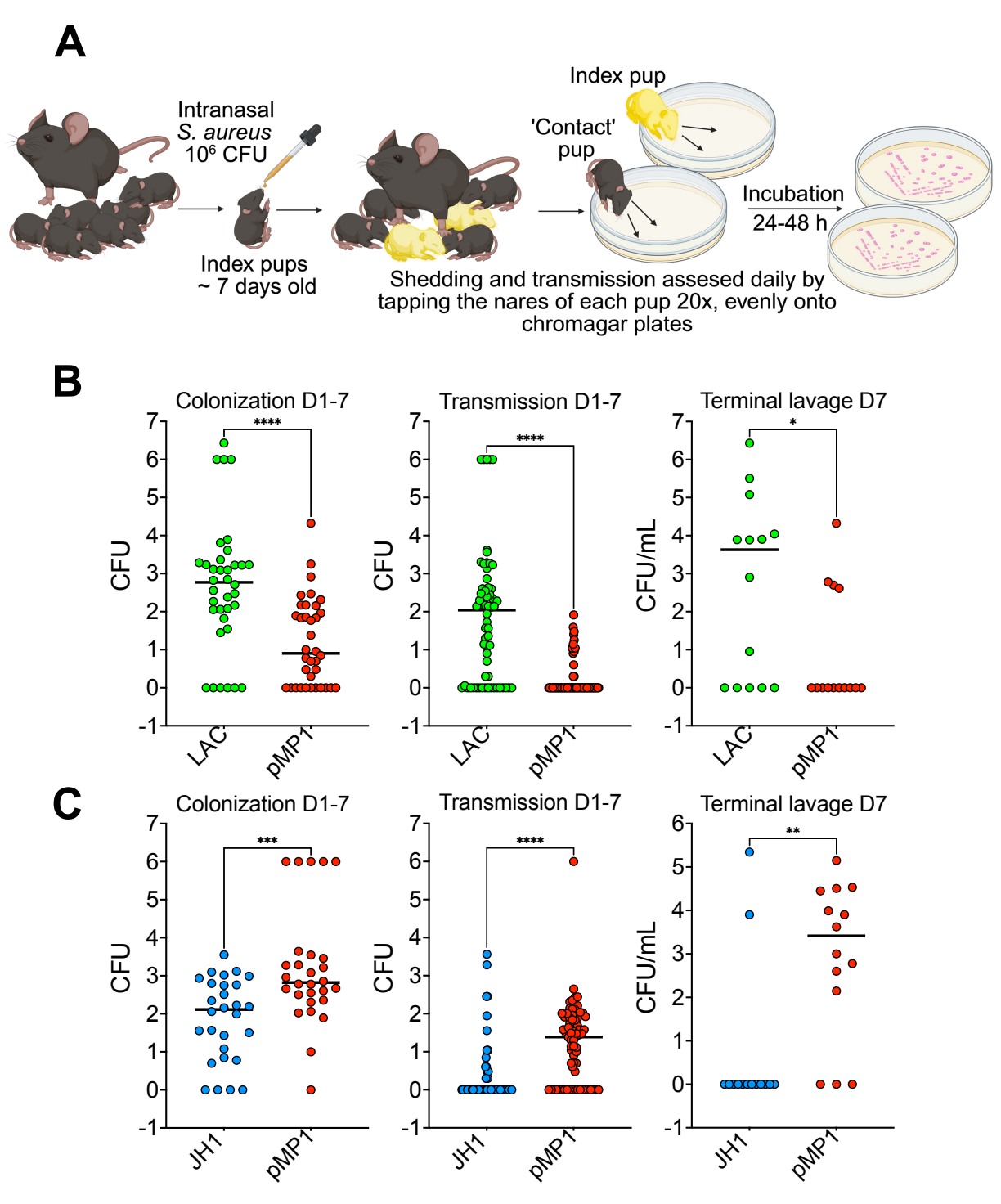
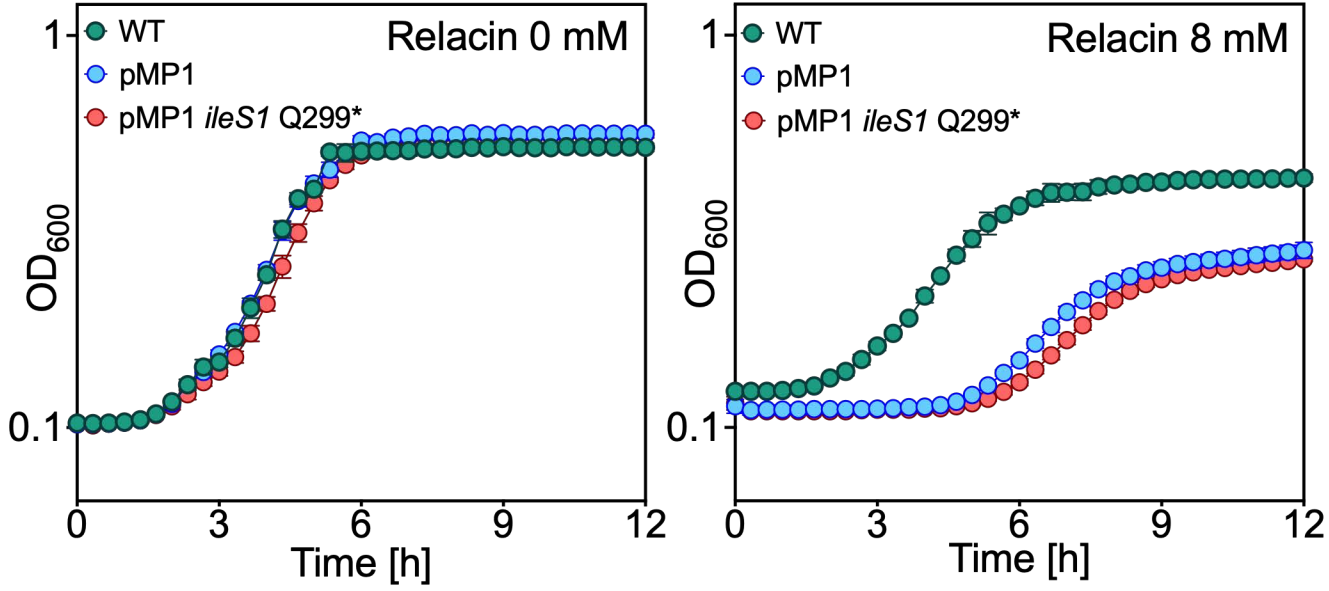
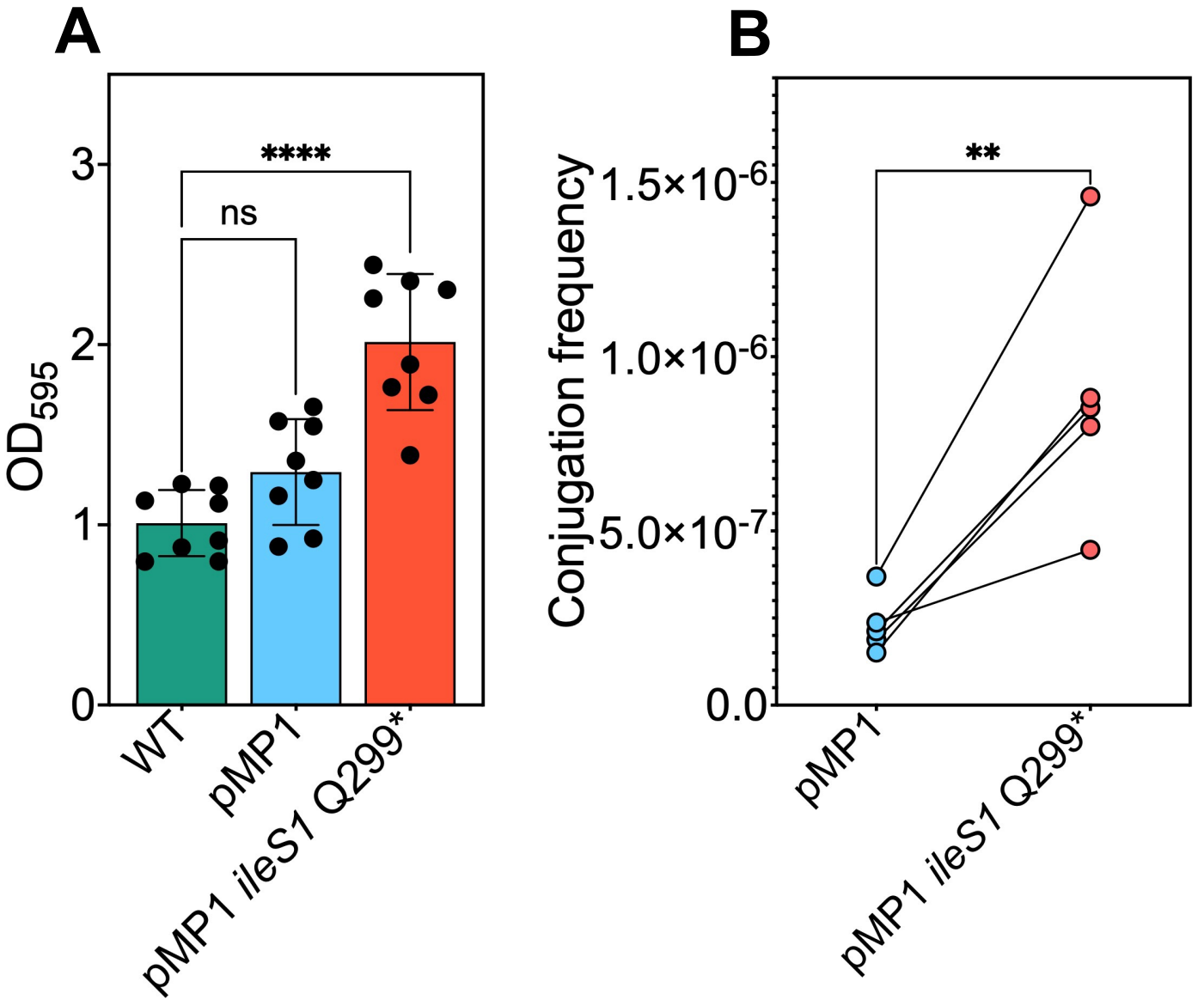
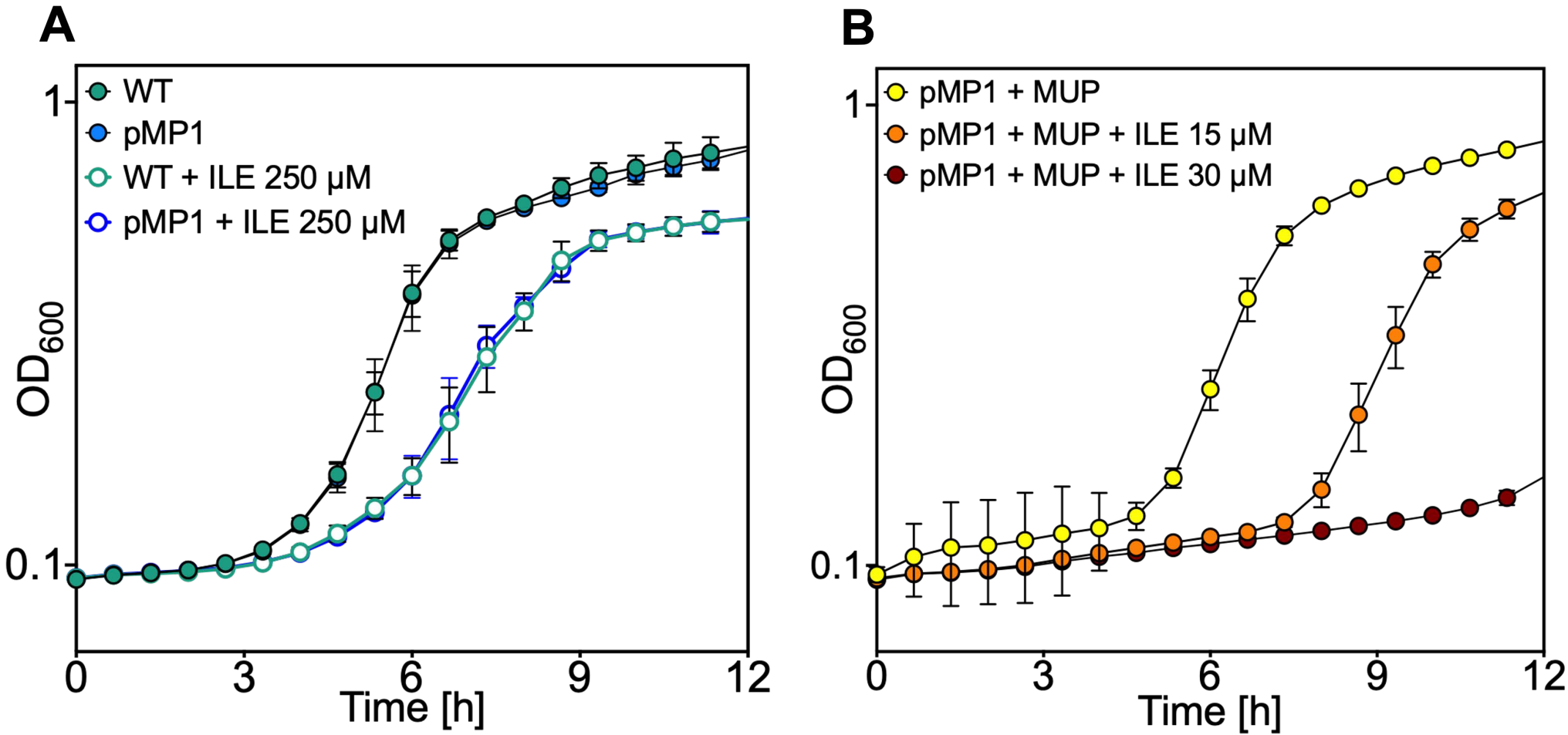
2
3
4
5
6
7
8
9
10
11
Novel
ileS1 variants
Truncation
Frame shift


A
Intranasal
S. aureus
10^6 CFU
Index pups
~ 7 days old
Index pup
'Contact'
pup
Incubation
24-48 h
Shedding and transmission assesed daily by
tapping the nares of each pup 20x, evenly onto
chromagar plates
B
Colonization D1-7
Transmission D1-7
Terminal lavage D7
CFU
CFU/mL
****
****
*
LAC
pMP1
C
Colonization D1-7
Transmission D1-7
Terminal lavage D7
CFU
CFU/mL
***
****
**
JH1
pMP1


WT
pMP1
pMP1 ileS1 Q299*
Relacin 0 mM
OD600
1
0.1
0
3
6
9
12
Time [h]
WT
pMP1
pMP1 ileS1 Q299*
Relacin 8 mM
OD600
1
0.1
0
3
6
9
12
Time [h]


A
B
****
ns
**
OD595
Conjugation frequency
3
2
1
0
1.5×10-6
1.0×10-6
5.0×10-7
0.0
WT
pMP1
pMP1 ileS1 Q299*
pMP1
pMP1 ileS1 Q299*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pMP1
WT + ILE 250 µM
pMP1 + ILE 250 µM
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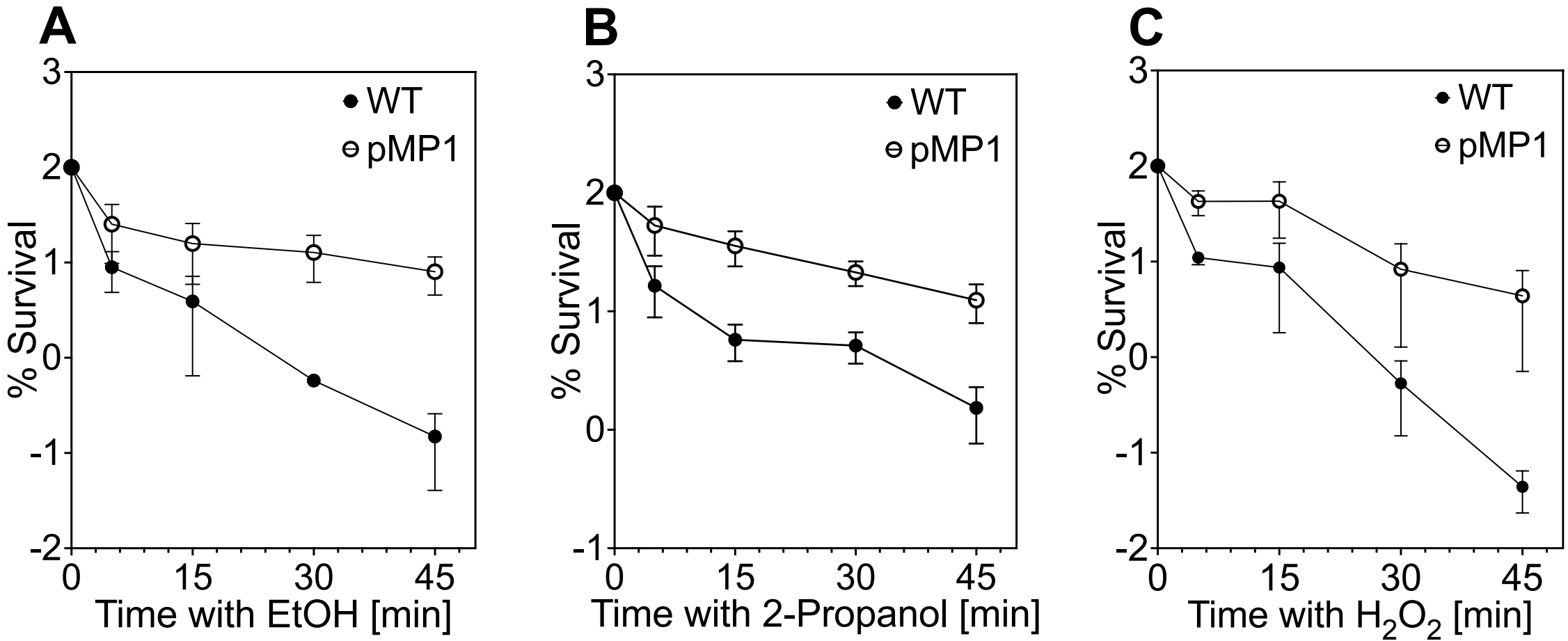
0.1
OD600
0
3
6
9
12
Time [h]
B
pMP1 + MUP
pMP1 + MUP + ILE 15 µM
pMP1 + MUP + ILE 30 µM
1
0.1
OD600
0
3
6
9
12
Time [h]


A
B
C
WT
pMP1
% Survival
Time with EtOH [min]
Time with 2-Propanol [min]
Time with H_2O_2 [min]
3
2
1
0
-1
-2
0
15
30
45