Epidemic MRSA clone ST22-IV is more resistant to multiple host- and environment-related stresses compared with ST228-I

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Background: ST22-IV is a successful hospital-associated MRSA clone. Due to its known ability to replace other MRSA clones in hospitals, it became a dominant clone in Europe and beyond. So far, there are no studies investigating the relationship between the epidemiological success of MRSA clones and their capacity to withstand commonly encountered stresses.

Methods: We investigated the fitness of ST22-IV in comparison with the replaced clone ST228-I, evaluating its resistance to oxidative stress, autolytic activity, growth at high osmolarity and in acid and alkaline environments and survival under desiccation and heat shock. We also compared their phenotypic characteristics and examined the impact of antibiotic consumption on epidemiological success.

Results: Here we demonstrate that the dominance of ST22-IV is linked neither to changes in antibiotic consumption nor to acquisition of additional resistances over time. Strong α-haemolysin activity, the production of β-haemolysin and the presence of an active agr could partly explain the virulence of ST22-IV previously observed in a murine model of pneumonia. Most importantly, we show that ST22-IV compared with ST228-I, besides retaining susceptibility to most antibiotics over time, has a superior capacity to survive under all stress conditions tested, which bacteria commonly face during their life cycle.

Conclusions: Our results support our hypothesis that ST22-IV has a fitness advantage over ST228-I. This fitness advantage could have allowed ST22-IV to displace ST228-I without acquiring additional resistances and could help explain its epidemic success in hospital settings and its spread in Europe and beyond.

Keywords: stress resistance, fitness, S. aureus

Introduction

The global spread of MRSA over the last 50 years represents a serious challenge to clinicians worldwide. North and South America and Asia report the highest prevalence rates of MRSA (>50%). China, Africa, Australia and some European countries, including Italy, have prevalence rates ranging from 25% to 50%. Other countries in Northern Europe show low prevalence rates.1 Seven European countries reported significant decreasing trends of invasive MRSA infections for the period 2009–12, mostly attributed to improved infection control measures. However, 7 out of the 30 reporting European countries, including Italy, reported MRSA percentages >25%, indicating that MRSA remains a public health priority.2

Some clones predominate in geographically limited regions, while others have disseminated worldwide.4 The majority of MRSA infections are caused by strains belonging to a few clonal complexes (CCs): CCs 5, 22 and 45 are predominant in hospital settings, while CCs 1, 59 and 80 are frequently isolated in the community.5-6 CCs 8 and 30 are pandemic in both hospital and community settings.7-11

The gentamicin-susceptible clone ST22-IV (CC22) has been recognized as the most successful and rapidly disseminating hospital-acquired (HA) MRSA clone in Europe and beyond.12,13 It was first isolated in the UK in the early 1990s.14 Afterwards it spread all over Europe, causing outbreaks also in the community, and replaced other clones.12,15-17 It became a dominant clone also in New Zealand, Australia and India.13,20 In Italy, we reported...
the displacement of the predominant Italian clone ST228-I (CC5) by ST22-IV. The reasons for the success of ST22-IV are still poorly understood. Holden et al. associated its pandemic dissemination to the introduction of fluoroquinolones in the 1980s and the emergence of fluoroquinolone resistance in this clone. However, the success of ST22-IV cannot rely only on the acquisition of fluoroquinolone resistance, as this is a phenotypic feature common to most HA MRSA clones, including the minor clones detected in our hospital, such as ST8-IV, ST5-II and ST5-IV.

We previously showed that ST22-IV was able to favourably modify its biofilm production over time and to outcompete ST228-I in co-culture. Thus, we hypothesized that ST22-IV could be fitter than ST228-I in the hospital environment. In this study we investigate the possible reasons for the difference in fitness by evaluating the impact of drug resistance, infection control policies and antibiotic consumption, by correlating the clones’ phenotypic characteristics to their pathogenicity and by investigating the relationship between the clones’ epidemic success and resistance to physical–chemical stresses encountered during the infectious process or in the environment.

Materials and methods

Bacterial strains

For this study, we selected all MRSA (n=465) belonging to the predominant clones ST22-IV (n=245) and ST228-I (n=220) from a collection of 693 non-duplicate strains, previously characterized by PFGE, MLST and drug susceptibility testing, as described elsewhere. These strains were isolated from mid-2006 to the end of 2011 from patients with MRSA infection (not colonization) admitted at the San Raffaele Hospital (OSR). OSR is a large (1400 beds) private university hospital located in the north-eastern area of Milan. Phenotypic characterization (haemolytic activity and δ-haemolysin production) was performed on 100 ST22-IV and 100 ST228-I strains. Stress experiments, including hydrogen peroxide susceptibility, autolytic activity, growth at high salinity and in acid and alkaline environments, and survival under desiccation and heat shock, were performed on 10 ST22-IV and 10 ST228-I strains. Strains were selected from the whole study period, considering the opposite isolation trends of the two clones (Figure 1d) and excluding strains isolated from patients identified as secondary cases due to nosocomial transmission. Each stress experiment was performed at least three times independently.

Antimicrobial susceptibility testing and antibiotic consumption

The antimicrobial resistance profile of all MRSA was determined using the Vitek-2 system (bioMérieux, Marcy-l’Étoile, France) and interpreted according to the CLSI guidelines, against a panel of 10 antimicrobial agents: levofloxacin, clindamycin, erythromycin, gentamicin, oxacillin, rifampicin, tetracycline, co-trimoxazole, teicoplanin and vancomycin. The macrolide–lincosamide–streptogramin B resistance phenotype was determined by the D-test, according to the CLSI. Strains were classified as MDR when they were non-susceptible to one or more agents in three or more antimicrobial categories, excluding β-lactams. Annual antibiotic consumption at the OSR was expressed as DDDs/100 patients/day to determine the percentage of patients exposed every day to antimicrobials.

Haemolytic activity and δ-haemolysin production

Haemolytic properties of strains were assessed as previously described. The Staphylococcus aureus strain RN4220, which produces a large zone of β-haemolysis without the interference of α- or δ-haemolysin, was used to evaluate the ‘hot–cold lysis’ associated with δ-haemolysin production. Strains RN6390 and Mu50 were used as positive and negative controls, respectively, for the production of δ-haemolysin.

Hydrogen peroxide susceptibility

Susceptibility to hydrogen peroxide was determined using the disc diffusion assay. A mid-log-phase bacterial culture (100 μL) was spread evenly on a Tryptic soy agar (TSA) plate and allowed to dry. A sterile 6 mm filter paper disc containing 10 μL of either 15% or 30% H2O2 was placed on the plate. Diameters of zones of inhibition of growth were measured in millimetres after 24 h of incubation at 37°C.

Autolysis

An autolysis assay was carried out as described by Singh et al. with minor modifications. Overnight-grown bacteria were subcultured in fresh Trypticose soy broth (TSB) and allowed to grow for 2 h at 37°C with shaking (200 rpm) to reach mid-exponential phase. After one wash with ice-cold PBS, cells were resuspended to an initial OD600 of 0.5 in 0.05 M Tris/HCl buffer, pH 7.2, containing 0.05% Triton X-100. The flasks were incubated at 37°C with shaking (200 rpm). The viability of bacteria was measured by subsequent OD600 readings every hour for 6 h.

Growth at high osmolarity

Bacteria were grown to mid-exponential phase (OD600 0.25–0.3) in TSB at 37°C and serially diluted, and 10 μL spots of each dilution were plated onto TSA with or without 2.2 M NaCl. After incubation at 37°C for 42 h, cfu were counted.

Desiccation survival

The capacity of MRSA strains to survive desiccation was evaluated following the method of Knight et al. The percentages of bacteria surviving at the end timepoints (6 h, 24 h, 5 days and 7 days) were calculated, as well as the average daily death rate at 1 day (K) using the following formula: \[ K = 2.3 \times \left(\frac{\log_{10} B_{x} - \log_{10} B_{0} - 1}{d}\right) \], where \( B_{x} \) is the log10 cfu at day x from the time of inoculation.

Thermotolerance assay

Overnight bacterial cultures were diluted in fresh TSB and placed at 37°C until the OD600 reached 0.3. The cultures were then divided into two ports: one was maintained at 37°C and the other was incubated at 48°C for 30 min. Both flasks were then shifted to 60°C, and after 0 and 8 min aliquots of the cultures were diluted in TSB, plated on TSA and incubated at 37°C for 48 h, followed by cfu counting.

Growth at acid/basic pH

Bacteria from mid-exponential phase cultures were washed with ice-cold PBS and inoculated in 20 mL of TSB, pH 4.0, or TSB, pH 10.0, at a concentration of 0.03 OD600. The two flasks were then incubated at 37°C and the OD600 was measured hourly for 6 h to monitor the growth rate.

Statistical analysis

Spearman’s correlation (rho) was used to assess the relationship between antibiotic consumption and the incidence of clones ST22-IV and ST228-I. Fisher’s exact test or the two-sided Mann–Whitney test was used to evaluate phenotypic differences between the two clones (e.g. antimicrobial resistance profiles, haemolytic properties and hydrogen peroxide susceptibility), depending on the type of response variable. Differences between clones in the stress experiments were assessed by using linear
mixed-effects (LME) or non-linear mixed-effects (NLME) models, which can account for the dependency structure among replicates and strains, and among the longitudinal measurements of the same replicate (see the Supplementary data, available at JAC Online). To consider the variability among replicates of the same strain and among clonal strains, nested random effects were set on the intercept term of the LME models and on the asymptote of the NLME model. To meet the assumptions of the model, an adequate transformation of the dependent variable was considered and normality tests (Royston and Shapiro–Wilk tests for longitudinal and non-longitudinal data, respectively) were performed by clone and replicate to ensure the normality of the data. In the non-longitudinal experiments, we used LME models with terms for testing differences between clones and/or between conditions, including an interaction term (between the two variables representing the conditions and the clones) when appropriate. For longitudinal experiments, all LME and NLME model parameters were tested in relation to the clone type and, when necessary, a backward selection procedure on the fixed-effects covariates was applied. The detailed formulae of all the estimated LME and NLME models are given in the Supplementary data.

In the study period, there was a significant increase in the percentage of strains with inducible resistance (22.5% versus 65.3%, P = 0.02). Over time, the percentage of rifampicin-resistant ST228-I strains significantly increased from 38.8% to 70.3% (P = 0.04). With the exception of clindamycin, ST22-IV did not acquire new resistances over the years.

For ST22-IV we identified 12 different antibiotic resistance profiles, 9 of them classified as non-MDR profiles and accounting for 31% of the strains. Two profiles included 87.1% of the strains: 66.2% of the ST22-IV strains were resistant to levofloxacin, erythromycin and clindamycin and 20.9% presented a non-MDR phenotype, being resistant only to levofloxacin (Table S1). For ST228-I we detected 11 MDR and only 2 non-MDR resistance profiles. Two MDR patterns accounted for 91.3% of the strains: 54.5% of the ST228-I strains were resistant to levofloxacin, erythromycin, clindamycin and gentamicin, and 36.8% were also resistant to rifampicin; only <2% of the strains showed a non-MDR phenotype (Table S1).

### Infection control measures and antibiotic consumption

In 2006, the infection control committee of the OSR drew up a 5 year plan (2007–11) for management and prevention of nosocomial infections. Several important strategies were implemented during the 5 year period: designation of infection control assistants in each ward; revision of the guidelines on hand hygiene, environment cleaning and antiseptic and disinfectant use; introduction of guidelines on antibiotic prophylaxis; and education and training activities of healthcare workers. Moreover, molecular typing of the major MDR organisms (MDROs) was introduced to rapidly identify and limit epidemic clusters. The guidelines on isolation and contact precautions were strengthened to control the spread of MDROs and reduce the risk of transmission. As all these measures were introduced to fight healthcare-associated infections and none of them specifically targeted MRSA, we could not define their contribution to the increase in ST22-IV and the decline in ST228-I.

Between 2006 and 2011 the incidence of MRSA in our hospital remained stable around 38%. Figure 1 presents data on antibiotic consumption and isolation trends for ST228-I, ST22-IV and other clones during the study period at the OSR. Other clones included...
ST8-IV and minor clones, such as ST5-II, ST5-IV and ST239-III, which showed a fluctuating isolation trend, excluding their competition with the predominant clones. Curiously, we observed a significant increase over time in the percentage of ST228-I and ST22-IV strains resistant to rifampicin and clindamycin, respectively, although between 2006 and 2011 the consumption of these antibiotics decreased by 10% and 58%, respectively (Figure 1c). The only significant relationship between antibiotic use and the incidences of the two clones was found for fluoroquinolones. Increased consumption of fluoroquinolones correlated positively with the increase in ST22-IV (rho = 1.00, 95% CI 1.00 to 1.00, P = 0.0028) and negatively with the decline in ST228-I (rho = -0.94, 95% CI -1.00 to -0.50, P = 0.0167) (Figure 1b and d).

**Haemolytic properties and activity of the global regulator agr**

The clones displayed strikingly different haemolytic properties. α-Haemolysin was detected in 98% and 55% of the ST22-IV and ST228-I strains, respectively (P < 0.0001). While ST22-IV strains presented a large zone of haemolysis, ST228-I strains were weakly haemolytic. β-Haemolysin was produced by 85% and 28% of ST22-IV and ST228-I strains, respectively (P < 0.0001). The biggest difference was related to the activity of the global virulence regulator agr: 92% of the ST22-IV strains and only 4% of the ST228-1 strains produced β-haemolysin, suggestive of the presence of an active agr (P < 0.0001).

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**Figure 1.** Antibiotic consumption and MRSA clone isolation trends at the OSR, 2006–11. Data on antibiotic consumption are presented as DDDs/100 patients/day for (a) β-lactams (cephalosporins, carbapenems and β-lactamase-resistant penicillins), (b) fluoroquinolones, macrolides, aminoglycosides and glycopeptides and (c) clindamycin, rifampicin, co-trimoxazole and tetracyclines. Cephalosporins include cefazolin, cefotaxin, cefotaxime, ceftazidime and ceftriaxone. Carbapenems include meropenem, imipenem and ertapenem. β-Lactamase-resistant penicillins include ampicillin/subactam, amoxicillin/clavulanic acid, ticarcillin/clavulanic acid and piperacillin/tazobactam. Fluoroquinolones include ciprofloxacin and levofloxacin. Macrolides include erythromycin, clarithromycin and azithromycin. Aminoglycosides include gentamicin, amikacin and tobramycin. Glycopeptides include vancomycin and teicoplanin. The percentages of MRSA strains belonging to ST228-I, ST22-IV and other clones isolated during the study period are also shown (d).
Epidemic MRSA clone ST22-IV is highly stress resistant

**ST22-IV was more resistant to hydrogen peroxide than ST228-I**

ST22-IV was more resistant than ST228-I to both concentrations of hydrogen peroxide tested, displaying a smaller median inhibition halo (15% H₂O₂, 32.5 and 37 mm, respectively, \( P = 0.0002 \); 30% H₂O₂, 38 and 42.5 mm, respectively, \( P = 0.0004 \)).

**ST22-IV had reduced autolytic activity compared with ST228-I**

Figure S1(a and b) shows the percentage of lysed bacteria at different timepoints and the estimated LME model of the data transformed on the logit scale, respectively. The intercept and the slope of the estimated curve were significantly different for the two clones (Figure S1b). From the first timepoint (1 h), ST228-I presented a higher proportion of lysed cells compared with ST22-IV (\( \beta_3, P < 0.0001 \)). As a consequence, the slope of the ST228-I lysis curve was significantly lower than that associated with ST22-IV: the percentage of lysed ST228-I bacteria increased more slowly over time compared with ST22-IV (\( \beta_3, P = 0.0091 \)). Thus, ST22-IV had significantly reduced autolytic activity compared with ST228-I.

**Osmotic stress had opposite effects on ST22-IV and ST228-I growth**

ST22-IV was particularly halotolerant, showing significantly increased growth in the presence of a high salt concentration compared with physiological conditions (Figure S2a, \( P < 0.0265 \)). In contrast, high salinity significantly reduced the growth of ST228-I (Figure S2a, \( P < 0.0001 \)). Our models estimated that, for ST22-IV and ST228-I, the number of cfu in the presence of salt was 1.13- and 0.76-fold, respectively, the number of cfu at physiological osmolarity. These data are in agreement with the analysis of the cfu percentage ratio (Figure S2b, \( P = 0.0002 \)). Both clones presented similar growth at physiological salt concentration (\( P = 0.4349 \)), while ST228-I displayed lower growth compared with ST22-IV at high osmolarity (Figure S2a, \( P < 0.0001 \)).

**ST22-IV had higher desiccation tolerance compared with ST228-I**

Figure 2(a and b) shows the percentage of the inoculum surviving desiccation over time and the estimated LME model of the natural logarithm of the data, respectively. The inoculum being equal for both clones, we observed that, compared with ST228-I, ST22-IV presented a higher percentage of bacteria surviving desiccation (Figure 2a). The intercept of the ST22-IV estimated curve was significantly higher than that associated with ST228-I, meaning that, at the first timepoint (6 h), ST22-IV showed a greater proportion of surviving bacteria compared with ST228-I (Figure 2b, \( \beta_1, P = 0.0035 \)). These data were further confirmed by the average death rate calculated at 24 h, which was significantly lower for ST22-IV compared with ST228-I (0.272 and 0.705, respectively, \( P = 0.0029 \)). The difference between the clones in the fraction of surviving bacteria was maintained until the last timepoint (7 days, Figure 2b).

**ST22-IV had superior heat resistance compared with ST228-I**

For both clones, incubation at 48°C, before exposure to 60°C, resulted in a significantly higher proportion of viable bacteria than pre-incubation at 37°C (Figure 3, \( P < 0.0001 \)). Transfer from physiological to lethal temperature (60°C) caused the death of almost all bacteria, with <0.5% of cells surviving for both clones.
In this case, ST228-I was associated with a slightly but significantly higher average percentage of viable cells compared with ST22-IV (0.039% versus 0.004%, \( P = 0.0004 \)). On the other hand, transfer from sublethal (48°C) to lethal temperature had a strikingly different effect on the two clones: ST22-IV, compared with ST228-I, showed a higher percentage of cells able to develop heat resistance and survive at lethal temperature (Figure 3b, 16.97% versus 0.92%, \( P < 0.0001 \)).

**Figure 3.** Viability after heat shock. The viability of ST22-IV and ST228-I clones after pre-incubation at either physiological temperature (37°C, a) or sublethal temperature (48°C, b) followed by exposure to lethal temperature (60°C) was determined by cfu counting. Each symbol represents the average percentage of surviving bacteria for a single MRSA strain. The average percentage of viable bacteria for each clonal group is indicated by a black horizontal line. LME models were used to evaluate the effect of heat shock on the growth of each MRSA clone and the differences between clones.

We previously showed that ST22-IV and ST228-I had similar growth rates in pure culture at physiological pH.\(^7\) Figure 4 (a and c) presents the growth rate of the clones at acid and basic pH, respectively. Figure 4(b and d) shows the corresponding NLME models of the natural logarithm of the growth rate. Compared with physiological pH, both clones grew more slowly in acid and alkaline environments (data not shown). However, at both pH values we observed that, during the exponential phase, ST228-I displayed a reduced growth rate compared with ST22-IV (Figure 4a and c). ST22-IV seemed to be less affected by and better adapted to pH changes. For each clone and pH condition, the NLME model analysis estimated the timepoints corresponding to half (inflection point) and 0.73 of the plateau (scale parameter) of the growth curve. These parameters were significantly higher for ST228-I compared with ST22-IV, indicating that ST22-IV grew faster than ST228-I (Figure 4b and d, \( \beta_2 \) and \( \beta_4 \), \( P < 0.05 \)).

**Discussion**

The factors associated with the epidemic success and the ability of ST22-IV to displace other clones in the healthcare setting are still unclear. Thouerez et al.\(^6\) demonstrated that fitness advantages are crucial in the dissemination of MRSA clones in hospitals. In the hospital environment there is strong selective antibiotic pressure and multidrug resistance in HA MRSA is common.\(^6\) Knight et al.\(^12\) observed a shift from CC30 and ST239 clones to CC22 over a 10 year period. They correlated this phenomenon with the ability of CC22 to acquire resistances and to shuffle them between isolates.\(^6,12\) In contrast, between 2006 and 2011 we noticed that ST22-IV did not accumulate resistances. Compared with ST228-I, ST22-IV retained susceptibility to erythromycin, gentamicin, rifampicin and clindamycin, and 31% of the ST22-IV strains were non-MDR, with only fluoroquinolone resistance being the second most represented phenotype. This is not surprising, as additional antibiotic resistances could represent a fitness cost. In agreement with Knight et al.,\(^12\) our data indicated that the replacement of ST228-I by ST22-IV was not associated with changes in antibiotic consumption in our hospital and that increased use of fluoroquinolones was a driving force for selection of successful HA MRSA clones. Although there was a strengthening of the infection control policies during the study period, the incidence of MRSA remained stable and we could not correlate these changes to the increase in ST22-IV and the decline in ST228-I.

We found a correlation between the different pathogenities of the two clones, previously observed in a murine model of
Pneumonia, and their haemolytic properties. It is known that α-toxin has an essential role in the pathogenesis of pneumonia and very recently it has been demonstrated that β-toxin production leads to the development of large caseous lesions in pneumonia. Moreover, δ-toxin is a member of the family of phenol-soluble modulins (PSMs), which, like α-toxin, are critical virulence factors. Only strains with dysfunctional agr lack PSM production. In agreement with its increased virulence observed in an animal model, ST22-IV, compared with ST228-I, was significantly associated with stronger α-haemolysin activity, the production of β-haemolysin and an active agr.

Our results showed that the capacity to survive under stress conditions could contribute to the selection of successful epidemic MRSA clones. S. aureus is resistant to a variety of environment- and host-related stresses, such as oxidative stress and changes in pH, osmotic pressure and temperature. Production of reactive oxygen species (ROS), including hydrogen peroxide, is one of the mechanisms adopted by phagocytic cells.
to kill bacteria. Oxidative stress resistance in S. aureus is mediated by detoxification enzymes. ST22-IV was significantly more resistant to hydrogen peroxide than ST228-I, indicating a superior capacity to neutralize ROS.

Autolysis, which is essential in cell wall turnover, can be triggered by antibiotics or adverse physiological conditions. Of concern, vancomycin-intermediate S. aureus (VISA) usually has a reduced autolysis rate and a thick cell wall. ST22-IV had a significantly reduced autolysis rate compared with ST228-I and better survived the attack on cell wall stability posed by Triton X-100.

S. aureus can grow at high NaCl concentrations (up to 3.5 M) accumulating within the cytoplasm osmoprotectants. In addition, the interpeptide bridges of cell-wall peptidoglycans are shortened to confer mechanical strength on the cell to resist drastic changes in osmotic pressure. High salinity determined a significant reduction in ST228-I growth. Surprisingly, the growth efficiency of ST22-IV at high osmolarity was significantly greater compared with physiological conditions. This hyper-tolerance could allow ST22-IV to predominate over other clones as a colonizer of high-salt environments, such as human skin or the mucous membrane of the anterior nares, thus favouring its transmission. Very recently Hart et al. demonstrated a significantly higher colonization rate of healthcare workers during ST22-IV outbreaks compared with ST239-III outbreaks.

S. aureus can survive in hostile environments outside the host as it is extremely desiccation tolerant. Compared with ST228-I, ST22-IV was more desiccation tolerant, showing a higher proportion of recovered viable bacteria and a lower death rate at 24 h. This could enhance its potential for persistence and dissemination in the hospital, with consequent transmission to patients.

Exposing bacterial cells to sublethal temperatures induces thermotolerance in various species, including Escherichia coli, Listeria monocytogenes, Yersinia enterocolitica and S. aureus. Our data indicated that ST22-IV compared with ST228-I, had a superior capacity to survive thermal stress and develop heat resistance.

To colonize, survive and cause infection, S. aureus must successfully adapt to transient pH variations due to natural or artificial circumstances. Compared with ST228-I, ST22-IV was less affected by and better survived drastic pH changes.

To the best of our knowledge, this is the first study that explores the correlation between the epidemiological success of an MRSA clone, specifically ST22-IV, and its capacity to survive under stressful conditions. We demonstrate that the success of ST22-IV is linked to neither changes in antibiotic consumption nor to acquisition of additional resistances. Most importantly, we show that ST22-IV, besides retaining susceptibility to most antibiotics over time, compared with ST228-I, has a superior capacity to survive under different stress conditions that bacteria commonly face during their life cycle inside the host and in the environment. Our results support our hypothesis that ST22-IV is fitter than ST228-1. This fitness advantage could have allowed ST22-IV to displace ST228-I without acquiring additional resistances and could help explain its epidemic success. Further studies analysing the proteome of these clones in the presence of different stressors should be undertaken to better understand the differences observed in this work in terms of viability and the molecular basis of the fitness advantage of ST22-IV. Extensive experiments investigating the colonization and transmission capacity of ST22-IV could allow fuller comprehension of its epidemiological success.

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References
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