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Signature-tagging of a bacterial isolate demonstrates phenotypic variability of the progeny *in vivo* in the absence of defined mutations

Paul W. Whitby^{a,*}, Timothy M. VanWagoner^{a,b}, Daniel J. Morton^a, Thomas W. Seale^a, Jennifer M. Springer^a, Randy J. Hempel^a, and Terrence L. Stull^{a,c}

^aDepartment of Pediatrics, University of Oklahoma Health Sciences Center, Oklahoma City, OK, 73104, USA

^bDepartment of Biology, Oklahoma Christian University, Oklahoma City, OK 73136, USA

^cDepartment of Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, 73104, USA

Abstract

Awareness of the high degree of redundancy that occurs in several nutrient uptake pathways of *H. influenzae* led us to attempt to develop a quantitative STM method that could identify both null mutants and mutants with decreased fitness that remain viable *in vivo*. To accomplish this task we designed a modified STM approach that utilized a set of signature tagged wild-type (STWT) strains (in a single genetic background) as carriers for mutations in genes of interest located elsewhere in the genome. Each STWT strain differed from the others by insertion of a unique, Q-PCR-detectable, seven base pair tag into the same redundant gene locus. Initially ten STWTs were created and characterized *in vitro* and *in vivo*. As anticipated, the STWT strains were not significantly different in their *in vitro* growth. However, in the chinchilla model of otitis media, certain STWTs outgrew others by several orders of magnitude in mixed infections. Removal of the predominant STWT resulted in its replacement by a different predominant STWT on retesting. Unexpectedly we observed that the STWT exhibiting the greatest proliferation was animal dependent. These findings identify an inherent inability of the signature tag methodologies to accurately elucidate fitness in this animal model of infection and underscore the subtleties of *H. influenzae* gene regulation.

Keywords

Haemophilus influenzae; Signature-tagged mutagenesis; Virulence

1. Introduction

High throughput methodologies allow the rapid determination of transcriptomes and proteomes yet provide little insight into the physiological role(s) of the individual genes/proteins of a microorganism. Thus, there is a need to develop high throughput

*Corresponding author: Paul W. Whitby, Department of Pediatrics, 940 S.L. Young Blvd. BMS340, Oklahoma City, OK 73118, USA, Tel: 405-271-6451, Fax: 405-271-5804, pwhitby@ouhsc.edu.

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methodologies to investigate the effects of defined bacterial mutations in animal models of infection. Signature tagged mutagenesis (STM) represents one such methodological approach. Originally described in 1995 (Hensel et al., 1995), STM has become firmly established as a technique to facilitate identification of essential genes. Since the first description, many bacterial species have been subjected to various modified STM studies to elucidate essential virulence factors (Mazurkiewicz et al., 2006). While STM based studies have elucidated many potential virulence determinants, the technique itself has been mostly limited to the identification of mutants that are non-viable in an experimental model. STM requires the individual construction of specific mutants and, as typically performed, can only determine the presence or absence of a given mutant strain in a defined population. This limits the methodological approach to the identification of null mutants unable to persist in the infected host, i.e. genes essential for survival in that environment. One goal of our ongoing research is to construct and to analyze an *H. influenzae* transposon library to identify genes with a potential role in virulence. Our ongoing studies seek to identify not just essential virulence determinants, but also mutations resulting in decreased fitness following challenge of an appropriate animal model with a mixture of strains. To achieve this, we further modified the STM protocol. Based on a previously described Q-PCR technique (Hunt et al., 2004) we proposed the creation of a series of constructs containing a specific signature tag (ST) adjacent to a selectable antibiotic marker replacing a redundant gene of *H. influenzae*. In doing so, a series of signature-tagged wild-type (STWT) isolates could be created that are identical with the sole exception of a seven nucleotide ST. Using Q-PCR, each STWT could not only be detected, but also quantified, both in mono-culture and in a mixed population with other STWT isolates. The STWT isolates would then act as identifiable carriers into which additional mutations could be incorporated and the effect of the mutation analyzed. In an individual experiment, 9 STWT each harboring a distinct secondary mutation (STWT-m) could be mixed with a single STWT serving as an internal control (STWT-c). Following inoculation of the 10 mixed strains in an appropriate animal model, samples could be collected and the impact of the secondary mutation on fitness could be assessed in the population by determining the ratio of each STWT-m to that of the STWT-c by Q-PCR. Thus, the impact of the secondary mutations on fitness could be rapidly assessed.

2. Methods

2.1 Bacterial Strains and Growth Conditions

H. influenzae Rd KW20 is the originally sequenced *H. influenzae* strain and was obtained from the ATCC (Fleischmann et al., 1995). *H. influenzae* nontypeable strain 86-028NP is a minimally passaged nasopharyngeal isolate from a pediatric patient who underwent tympanostomy and tube insertion for chronic otitis media at Columbus Children's Hospital, this strain has been genome sequenced and has also been extensively characterized in chinchilla models of otitis media (Bakaletz et al., 1999; Harrison et al., 2005; Kennedy et al., 2000; Suzuki et al., 1994). *H. influenzae* was routinely maintained on chocolate agar with bacitracin at 37°C or grown on brain heart infusion (BHI) agar supplemented with 10 µg ml⁻¹ heme and 10 µg ml⁻¹ β-NAD (supplemented BHI; sBHI) and the appropriate antibiotic(s). *Escherichia coli* TOP10 (Invitrogen) was used for cloning experiments and was routinely grown on LB agar supplemented with the appropriate antibiotics. Kanamycin was used at 25 µg ml⁻¹ in *H. influenzae* and 50 µg ml⁻¹ in *E. coli*.

2.2 Construction of signature tagged wild-type isolates

A series of mutagenic constructs were made, each of which contain a unique signature tag (ST) adjacent to a kanamycin resistance cassette. Individual ST sequences were selected from those described in a previous study (Hunt et al., 2004) as described in section 2.3. ST

constructs were made until 10 had been verified and optimized as described below in section 2.3. Each ST construct was created as follows: A set of primers were designed to amplify the upstream region of the kanamycin marker from Tn903. The 5' end of these primers included a *Bam*HI restriction site, and the unique 7 nucleotide ST region. Each of these primers is denoted with the prefix ST and a number relating to that specific tag, followed by the suffix "KANFWD". These primers were individually used in a PCR with a second primer targeting the downstream region of the kanamycin marker (SIGKAN-REV). This latter primer also contained a terminal *Bam*HI site. The resulting amplified product(s) were identical to each other with the exception of the unique 7-bp ST region upstream of the kanamycin resistance gene. Each product was ligated in to the TA-cloning vector pCR2.1-TOPO (Invitrogen) and verified by sequencing and Q-PCR analysis of the inserted DNA. Following verification, each construct was digested with *Bam*HI to excise the ST-kan insert which was subsequently ligated in to *Bam*HI linearized pC1C4 (Morton et al., 1999). The plasmid pC1C4 was originally designed as a mutagenic construct to delete the hemoglobin-haptoglobin binding protein gene *hgpC* (locus number HI0712 in strain Rd KW20), and comprises abutting ~1-kbp fragments corresponding to the upstream and downstream regions of HI0712 with a unique *Bam*HI site between them (Morton et al., 1999). Thus, the final construct(s) produced herein resulted in the insertion of the ST-kan DNA into the unique *Bam*HI site between the upstream and downstream regions of HI0712. Following verification, the constructs were used to transform competent *H. influenzae* strains to kanamycin resistance, thereby deleting the redundant HI0712 (*hgpC*) gene and replacing it with the unique ST-Kan construct, it should be noted that deletion of HI0712 has no impact on either *in vitro* growth or *in vivo* virulence in multiple strains (Morton et al., 1999; Morton et al., 2004a; Seale et al., 2006). Correct insertions were verified by PCR and sequencing. Primer sequences are listed in Table 1 (sequences are given for only the 10 STs that were selected based on the optimization described below).

2.3 Optimization and selection of signature tags

As each construct was finished it was tested for specificity in the Q-PCR using isolated plasmid as the target. Each Q-PCR utilized a common primer inside the kanamycin cassette (QPCR-STCOM2) and a distinct second primer designed to target the individual signature tags (primers have the prefix QPCR- followed by the specific tag they are designed to amplify). Constructs exhibiting cross reaction with any other ST construct were discarded at this point. Following transformation of constructs into *H. influenzae*, individual STWT strains were checked for linearity of amplification with the specific primer set as well as cross reactivity with the other primer sets. Any STWT strain that gave an amplification product with a primer pair other than the one specific to the ST was discarded from the study. Through this process a pool of 10 specific STWT isolates were confirmed. Primers used in the Q-PCRs are detailed in Table 1.

2.4 Quantitative real-time PCR

Quantitative real-time PCR (Q-PCR) was performed as previously described (VanWagoner et al., 2004) with smaller reaction volumes but increased replicates. In short, quantification reactions at each timepoint were performed in quadruplicate. Each quantification reaction was composed of 5 ul of SYBR Green PCR Master Mix (Applied Biosystems), 250 nM each primer, and 2.5 ul of template DNA. Quantification of the ratio of each STWT was determined using the $2^{-\Delta\Delta C_t}$ method of Livak and Schmittgen (Livak et al., 2001), using concurrently run STWT13 as a normalizer. Oligonucleotide primers were designed using Primer Express 2.0 (Applied Biosystems) and were tested to determine the amplification specificity, efficiency and linearity of the amplification with DNA concentration.

2.5 Growth Studies with *H. influenzae*

Growth curves were performed using the Bioscreen C Microbiology Reader as previously described (Morton et al., 2005; Morton et al., 2006; Whitby et al., 2006). *H. influenzae* were grown for 12 to 14 hours on chocolate agar with bacitracin, and used to inoculate 10 ml sBHI cultures that were incubated for 4 hours at 37°C with shaking (175 rpm; Lab-Line, Enviro Shaker). The 4 hour cultures were pelleted by centrifugation, washed once in PBS containing 0.1% (w/v) gelatin and resuspended to an optical density at 605 nm of 0.5 (Shimadzu UV-1201S spectrophotometer) in the same buffer. One milliliter of the bacterial suspension was diluted in 5 ml of 0.1% w/v gelatin in PBS, and the final bacterial suspension was used to inoculate fresh sBHI (0.1 % v/v inoculum to give an approximate initial concentration of 200,000 cfu ml⁻¹). Growth curves were performed in 300 µl volumes with five replicates for each growth condition in each individual experiment. Experiments were performed at least twice. Optical density measurements were taken at 600 nm at thirty-minute intervals with the Bioscreen C set to incubate at 37°C with constant shaking (machine setting “low”).

A second experiment was performed to assess growth of each STWT strain in a mixed culture. Standard inocula of each isolate were prepared as described above, and were used to inoculate a 60 ml culture of sBHI with equal numbers of each STWT strain. Samples were taken immediately after inoculation and at 1, 2, 4, 6, 8, 10 and 18 hours post inoculation for Q-PCR analysis. For each sample, the ratio of each STWT to STWT13, an arbitrarily chosen internal control, was determined.

2.6 Chinchilla model of otitis media

Animal studies were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and were approved by the Institutional Animal Care and Use Committee of the University of Oklahoma Health Sciences Center. Cohorts of adult chinchillas (*Chinchilla lanigera*) with no evidence of middle ear infection by either otoscopy or tympanometry upon enrollment in the study were used in three separate experiments as described below. Animals were rested for at least 7 days upon arrival to acclimate them to the vivarium prior to infection. Animal procedures have been described in detail elsewhere (Bakaletz et al., 1997; Bakaletz et al., 1999; Morton et al., 2012).

In the first experiment, three chinchillas were infected in both ears with a mixture containing approximately 10⁴ cfu of each of the 10 selected STWT derivatives of strain Rd KW20 (total challenge dose 10⁵ cfu). On days 1, 2, 3 and 4 post challenge middle ear effusions (MEE) were collected by epitympanic tap.

In the second experiment, two chinchillas were infected in both ears with a mixture containing approximately 10⁴ cfu of each of the 10 selected STWT derivatives of strain 86-028NP (total challenge dose 10⁵ cfu). On days 4, 7 and 11 post challenge middle ear effusions (MEE) were collected by epitympanic tap.

In the third experiment, a further two chinchillas were infected in both ears with a mixture containing approximately 10⁴ cfu of each of the 10 selected STWT derivatives of strain 86-028NP (total challenge dose 10⁵ cfu). In this experiment however, each STWT had been recovered from the previously infected chinchilla middle ears at least 7 days post infection and had been subcultured only once post isolation. On days 4 and 7 post challenge middle ear effusions (MEE) were collected by epitympanic tap.

For all three experiments the actual challenge dose was confirmed by plate count and the ratio confirmed by Q-PCR of the individual sig tags in the inoculum. All recovered MEE

were immediately frozen to preserve the nucleic acid content of the sample prior to Q-PCR. Bacterial titers in MEE were determined using the track dilution method as previously described (Jett et al., 1997; Morton et al., 2004b).

2.7 Isolation of DNA from Chinchilla ear effusions

Chinchilla effusion samples (20ul) were diluted 1:10 and boiled for 10 minutes, followed by extraction using 1 volume phenol:chloroform:isoamyl alcohol (pH 6.2). The aqueous phase was removed and the DNA precipitated with 0.6x volumes isopropanol, 0.1x volume 3M sodium acetate, and 45 µg GlycoBlue (Life Technologies). The resulting pellet was washed once in 70% ethanol, dried, and resuspended in 200 µl of nuclease-free water prior to analysis by Q-PCR.

2.8 Statistics

Statistical comparisons of growth between strains under the same growth conditions *in vitro* were made using the Mann-Whitney test. Analyses were performed using Analyze-It for Microsoft Excel v1.71 (Analyze-It Software Inc., Leeds, England). A *P* value < 0.05 was taken as statistically significant.

3. Results

3.1 Selection of Signature tagged wild-types (STWT)

We initially constructed a series of vectors containing specific 7-nucleotide STs and a kanamycin resistance marker. Individual tags were tested for cross reactivity using the primers specific for each individual tag. Any ST that failed to amplify with its own specific primers, or was cross-reactive with any of the other primers was excluded from the study. In this fashion, a set of 10 ST constructs was identified that satisfied our selection criteria. They were tags 7, 8, 10, 11, 12, 13, 15, 17, 25, and 26, based on the previously described sequence numbering (Hunt et al., 2004). Once these 10 unique usable STs were identified, each was ligated into the pC1C4 vector (Morton et al., 1999), and the resulting vector was used to transform *H. influenzae* to kanamycin resistance. The correct insertion of the ST-kanamycin cassette, resulting in the deletion of the HI0712 (*hgpC*) gene, was confirmed by PCR for each ST construct. Once inserted into the *H. influenzae* genome the specificity of the STs was again determined as well as the linearity of amplification and detection limits of each ST. Each individual STWT could be specifically detected and also showed linearity of amplification with increased concentration from a baseline level of 100 CFU. Below this level, linearity was lost although as few as 50 bacterial cells could be detected in a sample. To ensure that linearity and detection limits were unaffected when in a mixed culture, the experiments were repeated in samples containing two STWT one of which was present at a thousand-fold excess of the other. No loss of linearity or detection was observed with any of the STWTs under these circumstances. Thus, each ST was deemed uniquely identifiable in a mixed culture and the level of target could be directly compared between tags.

3.2 Growth and detection of STWT strains in vitro

Since the proposed methodology requires each STWT to have identical growth kinetics, growth curves were performed with each individual STWT as well as the corresponding untagged wildtype strain. Each of the Rd KW20 derived STWT strains grew similarly to the other STWTs as well as the parent strain (Figure 1). Similar results were obtained for growth of the 86-028NP derived STWT strains (data not shown).

Having established that each STWT showed similar growth profiles in monoculture, experiments were performed to determine if the same was true in a culture containing all of the STWT strains. Samples were taken from a culture inoculated with equal numbers of each

STWT at 0, 1, 2, 4, 6, 8, 10 and 18 hours post inoculation and the relative prevalence of each STWT was determined by Q-PCR. For each sample, the ratio of the STWT to the experimental wild-type isolate (STWT13) was determined. Initially, each sample was normalized to the STWT13 data at that time point, and then the fold relative abundance of each STWT determined by comparison with the T=0 data. The relative fold changes from a representative experiment using the STWT derivatives of Rd KW20 are shown in table 2. The maximal variation between isolates, in a mixed culture is approximately 4-fold, and the majority of the samples are within 1.5-fold of STWT13. These data show that the isolates grow equally well in mixed culture *in vitro* and that there is no apparent competition between them in this environment. Similar results were obtained for growth of the 86-028NP derived STWT strains (data not shown).

3.3 Detection of STWT strains *in vivo*

Having demonstrated the utility of the STWT isolates *in vitro*, we sought to test their utility *in vivo*. Initially 4 chinchillas were infected in both ears with a mixture containing equal numbers of each of the ten STWT derivatives of Rd KW20. Epitympanic taps were attempted on all ears on days 1–4 post infection to collect MEE. MEE were processed and analyzed by Q-PCR to determine the ratio of each individual STWT relative to STWT13 present in each sample. Five of the infected ears yield recoverable MEE over the course of the experiment. Table 3 shows the ratio of STWT strains both in the inoculum and in each recovered MEE, and demonstrates that certain STWT strains grew better than other STWTs in individual animals. For example, in animal 1 STWT17 was present at between 15 and 58 fold greater levels than STWT13 (which was used as a normalizer) in all recovered MEE. Similarly, in animal 1 STWT25 was present at between 8 and 22 fold greater than STWT13 in 3 of 4 recovered MEE. All other STWT were present at significantly lower levels than STWT in all MEE recovered from animal 1, with STWT15 being undetectable in two MEE samples recovered from the right ear of this animal. In MEE recovered from chinchilla 2 the dominant isolate was STWT12, while in MEE from chinchilla 3 both of the isolates STWT11 and STWT25 were almost cleared.

These results were confirmed using the STWT derivatives of the *H. influenzae* isolate 86-028NP. As for the Rd KW20 STWT, inoculation of the ten 86-028NP mixed tags into the chinchilla middle ear selected for individual isolates in individual animals, with 86-028NP STWT12 dominating and STWT10 being cleared. In an attempt to correct for potential phase variation in the pool of 86-028NP STWTs, each individual STWT was isolated from a MEE recovered from an infected chinchilla at least 7 days post inoculation. The *in vivo* experiment was then repeated using the passaged STWTs to make the inoculum mixture. As before certain STWT strains, in this case STWT12 and STWT17, outgrew the other STWTs in individual animals (data not shown).

4. Discussion

These results highlight an inherent issue potentially affecting screening methodologies based on multi-isolate fitness. Prior to this study, we had assumed that identical wild-type isolates would survive and proliferate equally well in both broth and the chinchilla ear. Such is the basic assumption of signature tagged mutagenesis (Hensel et al., 1995). In this study we show that the tagged wild-types grow at a similar rate in a non-selective broth culture. However, this is not the case when the isolates are inoculated into a selective environment *in vivo*. The results from the tagged Rd KW20 and tagged 86-028NP isolates in the chinchilla ear suggest that this *in vivo* environment selects for certain isolates, even though the difference between them is only a seven nucleotide tag. Our data also show that the dominant isolate(s) differs between animals. Our initial studies in chinchillas utilized non-passaged isolates, although each STWT was created from the same parental stock. Since all

the animals received the same inoculum, it is reasonable to assume that individual isolates are varying in their respective fitness in individual animals. In Table 3, the data from 5 ears demonstrate a difference of several log magnitude between abundance of isolates. While this sample set is small, similar results were observed in repeat experiments and thus provide strong evidence for selective host-pathogen interactions. It is known that *H. influenzae* has a large array of phase variable elements (van Belkum et al., 1997). Although all the STWTs were derived from the same stock of Rd KW20 or 86-028NP it is possible that somatic changes in the phase variable regions have occurred between isolates. In the past we have recorded frequencies of phase variation up to 1 % for a one tetranucleotide repeat region (Ren et al., 1999). When the number of potentially phase variable loci and the number of total generations are considered, phase variation may have occurred resulting in a heterogeneous population. Overall, these data suggest that presumed clonal isolates can show a great degree of variation in general fitness *in vivo*. In addition to genomic phase variation, epigenetic differences may exist between the isolates in the inoculum that are propagated as individual bacterial cells colonize and begin to compete for available nutrients. Certain pathogens such as *Pseudomonas aeruginosa* adapt to a pathogenic lifestyle. Indeed, a positive STM has been developed to investigate this phenomenon (Bianconi et al., 2011). While the results we present raise questions as to the validity of data from STM, or large scale *in vivo* transposon studies, they may open the door to positive selection studies to investigate the process of *in vivo* adaptive selection. This phenomenon also could be of considerable importance to organisms such as *H. influenzae*, (of which the only known environmental niche is man) in the transition from harmless commensal to virulent pathogen.

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Highlights

- 10 uniquely tagged wildtype isolates of *Haemophilus influenzae* were created.
- In vitro, all 10 tagged isolates grew equally well.
- In vivo, individual tagged wild types showed differing growth kinetics.
- Individual chinchillas select individual tagged wild types.
- Phase variation and epigenetic mechanisms may be involved.

Figure 1.

Growth of the wild-type *H. influenzae* strain Rd KW20 and each of ten STWT strains in supplemented brain heart infusion broth. Solid circle, RdKW20; open circle STWT 7, solid square, STWT 10; open square, STWT 11; solid triangle, STWT 12; open triangle, STWT 13; solid diamonds, STWT 15; open diamonds, STWT 17; cross, STWT 25, small circle, STWT 26; star, STWT 8. For clarity, error bars are omitted. Values are mean $\pm$ SD for quintuplicate results from a representative experiment. The Mann-Whitney test was used to compare growth of all the strains over the entire growth period. There were no significant differences in growth between any pair of strains.

Table 1

Oligonucleotide sequences of primers used in this study.

Primer	Sequence
SIG7-KANFWD	CGGCCGGATCCGTACCGCGCTTAATGTAACCGAGGTTCTAGATTGTGTCTCAAAATCTCTGATG
SIG8-KANFWD	CGGCCGGATCCGTACCGCGCTTAAATCTCGGAGGTTCTAGATTGTGTCTCAAAATCTCTGATG
SIG10-KANFWD	CGGCCGGATCCGTACCGCGCTTAACAATCGTGAGGTTCTAGATTGTGTCTCAAAATCTCTGATG
SIG11-KANFWD	CGGCCGGATCCGTACCGCGCTTAATCAAGACGAGGTTCTAGATTGTGTCTCAAAATCTCTGATG
SIG12-KANFWD	CGGCCGGATCCGTACCGCGCTTAACTAGTAGGAGGTTCTAGATTGTGTCTCAAAATCTCTGATG
SIG13-KANFWD	CGGCCGGATCCGTACCGCGCTTAAACGTTTCAAGAGGTTCTAGATTGTGTCTCAAAATCTCTGATG
SIG15-KANFWD	CGGCCGGATCCGTACCGCGCTTAATACTGGAGAGGTTCTAGATTGTGTCTCAAAATCTCTGATG
SIG17-KANFWD	CGGCCGGATCCGTACCGCGCTTAAATGCAGAGAGGTTCTAGATTGTGTCTCAAAATCTCTGATG
SIG25-KANFWD	CGGCCGGATCCGTACCGCGCTTAATGAACTCGAGGTTCTAGATTGTGTCTCAAAATCTCTGATG
SIG26-KANFWD	CGGCCGGATCCGTACCGCGCTTAAGGTATCAGAGGTTCTAGATTGTGTCTCAAAATCTCTGATG
SIGKAN-REV	CGGCCGGATCCTCGGTCTGCGTTGTCGGAAGATGC
SPEC-C1C4	TGCAAATGCAGATTGTTCTACA
INSPEC1	GGCACTTACTAATTCCTCTATCAG
KANSIG-OUT	CATAAATCCGTCAGCCAGTTTAGTCTGACC
QPCR-ST7	GTACCGCGCTTAATGTAACCG
QPCR-ST8	GTACCGCGCTTAAATCTCGG
QPCR-ST10	GTACCGCGCTTAACAATCGTG
QPCR-ST11B	GTACCGCGCTTAATCAAGACG
QPCR-ST12B	GTACCGCGCTTAACTAGTAGG
QPCR-ST13B	GTACCGCGCTTAAACGTTTCAAG
QPCR-ST15B	GTACCGCGCTTAATACTGGAG
QPCR-ST17	GTACCGCGCTTAAATGCAGAG
QPCR-ST25	GTACCGCGCTTAATGAACTCG
QPCR-ST26	GTACCGCGCTTAAGGTATCAG
QPCR-STCOM2	ATGGCTCATAACACCCCTTGTATTA

Table 2Ratios of ST tagged wt strains following *in vitro* growth

STWT	0*	Time (hours)						
		1	2	4	6	8	10	18
7	1.00	1.44	1.76	2.37	2.22	1.57	1.35	2.15
8	1.00	0.93	0.97	1.03	1.29	0.98	1.02	0.74
10	1.00	1.27	1.22	1.07	1.52	1.09	1.05	0.84
11	1.00	1.08	0.99	0.99	1.48	1.46	1.55	0.90
12	1.00	0.99	0.98	0.99	1.3	1.22	1.22	0.80
13	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
15	1.00	1.00	0.97	1.03	1.19	1.11	1.16	0.75
17	1.00	0.92	0.76	0.46	0.41	0.41	0.37	0.24
25	1.00	1.12	0.89	0.76	0.76	0.67	0.68	0.35
26	1.00	0.82	0.73	0.58	0.57	0.45	0.54	0.35

* Values are derived by the $2^{-\Delta\Delta CT}$ method with normalizing to ST13 for each time point and then to the respective STWT strain at the initial time point (T=0).

Table 3

Ratios of ST tagged wt strains following *in vivo* growth

STWT	Inoc ⁺	Inoc [*]	Chinchilla ear sample									
			1LD2 ^Δ	1LD3	1RD2	1RD3	2LD2	2LD3	3LD3	3LD4	3RD3	3RD4
7	12600	1.00	0.03	0.27	0.23	0.06	0.01	0.07	0.38	0.72	0.01	nd
8	14467	1.00	0.02	0.03	0.10	0.05	0.03	0.02	0.01	0.02	0.04	0.01
10	14400	1.00	0.06	0.12	0.63	0.14	0.20	0.20	0.24	0.62	0.40	0.07
11	8533	1.00	0.09	0.16	0.10	0.05	0.08	0.04	0.01	0.01	0.05	0.01
12	9600	1.00	0.08	0.21	0.09	0.09	2.22	17.6	0.38	0.88	0.32	0.10
13	12783	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
15	9467	1.00	0.07	0.01	nd	nd	0.05	0.10	0.15	0.30	0.73	0.15
17	12800	1.00	32.60	58.74	19.54	14.72	1.97	0.96	0.33	0.29	0.50	0.79
25	11600	1.00	0.87	7.89	14.22	21.76	0.03	0.33	0.01	0.01	0.01	0.01
26	13861	1.00	0.20	0.14	nd	0.14	0.39	0.51	0.30	0.14	0.21	0.05

⁺Values in total CFU in the inoculum^{*}Values are derived by the $2^{-\Delta\Delta CT}$ method with normalization to ST13 for each chinchilla ear and then to the respective STWT strain from the initial inoculum.^ΔIndividual ears are labeled by the animal (denoted by the first number) the ear (denoted by either L for Left, or R for right) and the day number (denoted by Dn where n is the number).