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Competence for Genetic Transformation in *Streptococcus pneumoniae*: Mutations in σ^A Bypass the *comW* Requirement

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Competence for genetic transformation in the genus *Streptococcus* depends on an alternative sigma factor, σ^X, for coordinated synthesis of 23 proteins, which together establish the X state by permitting lysis of incompetent streptococci, uptake of DNA fragments, and integration of strands of that DNA into the resident genome. Initiation of transient accumulation of high levels of σ^X is coordinated between cells by transcription factors linked to peptide pheromone signals. In *Streptococcus pneumoniae*, elevated σ^X is insufficient for development of full competence without coexpression of a second competence-specific protein, ComW. ComW, shared by eight species in the *Streptococcus mitis* and *Streptococcus anginosus* groups, is regulated by the same pheromone circuit that controls σ^X, but its role in expression of the σ^X regulon is unknown. Using the strong, but not absolute, dependence of transformation on *comW* as a selective tool, we collected 27 independent *comW* bypass mutations and mapped them to 10 single-base transitions, all within *rpoD*, encoding the primary sigma factor subunit of RNA polymerase, σ^A. Eight mapped to sites in *rpoD* region 4 that are implicated in interaction with the core β subunit, indicating that ComW may act to facilitate competition of the alternative sigma factor σ^X for access to core polymerase.

Streptococcus pneumoniae is a common human pathogen, usually carried asymptomatically in the nasopharynx. A prominent characteristic of the bacterium is a natural ability to take up exogenous DNA from its environment. If the exogenous DNA is integrated into the genome, the bacterium is said to be transformed. Such transformation depends on development of a specialized physiological state, termed competence, development of which is coordinated within a culture by a quorum-sensing (QS) mechanism encoded by two genetic loci, *comAB* (1) and *comCDE* (2). Both loci are transcribed at a basal level by the "housekeeping" sigma factor, σ^A (3, 4). The competence-stimulating peptide (CSP), a product of the *comC* gene, is secreted by an ABC transporter/protease encoded by *comA* and *comB* (4, 5). CSP is sensed by a histidine kinase receptor, ComD, which phosphorylates a cognate response regulator, ComE (1, 4, 6, 7). Phosphorylated ComE activates the promoters of eight operons comprising 13 genes transcribed specifically at competence by binding a direct repeat centered at −40, the ComE box (4, 8, 9). They are designated early competence genes (9) and include both the *comAB* and *comCDE* operons (10–13). This organization creates a positive-feedback loop, ensuring a rapid increase in the level of CSP that can cause all the cells in a culture to become competent simultaneously (7, 13). One additional early gene essential for competence is *comX*, encoding the alternative sigma factor σ^X, which accumulates to high levels in response to CSP (14), forming a holoenzyme with RNA polymerase (RNAP) and enabling recognition of a noncanonical promoter sequence termed the ComX box, or cinbox, upstream of 23 genes, termed the late competence genes (13–16).

Streptococci have only one known alternative sigma factor, which in all species is linked to expression of the late competence genes. In the *Streptococcus mitis* and *Streptococcus anginosus* groups of species, upstream regulators of *comX* include CSP/ComABCDE QS systems orthologous to the pneumococcal one (17). In all other groups of streptococci, *sigX* is regulated by a different class of peptide QS circuit, using genes named *comS* and *comR*, encoding a peptide pheromone and intracellular receptor, respectively (17). Regulation of σ^X activity is important, as competence genes encode one or more killer proteins and cognate immunity genes. Within the *S. mitis* and *S. anginosus* groups, eight related species (*S. anginosus*, *Streptococcus cristatus*, *Streptococcus infantis*, *S. mitis*, *Streptococcus oligofermentans*, *Streptococcus oralis*, *S. pneumoniae*, and *Streptococcus pseudopneumoniae*) add another layer of control of σ^X activity in the form of a small protein, ComW, which plays an important role during competence development. Since its identification in 2004 in *S. pneumoniae* (18), attempts to identify the role of ComW have been reported (18–21), but its function remains unclear. *comW* mutants have several phenotypes that suggest different possible roles for the protein. First, *comW* mutants are ~10^4-fold deficient in transformants (18). Second, in *comW* mutants, transcription of late competence genes is strongly reduced, to ~10% of wild-type (WT) levels, and accumulation of σ^X protein is similarly reduced (19). Third, *comW clpE* (where ClpEP is a protease that degrades σ^X) double mutants produce restored amounts of σ^X protein but are still deficient in transformation (18). Fourth, both the N and C termini of ComW are necessary for σ^X stability, while the N terminus is necessary for σ^X function (18). Since none of the roles of ComW are well characterized and available data suggest several alternative roles during transformation, ComW's function in supporting σ^X activity remains unknown.

To investigate the role of ComW in regulation of competence in an unbiased manner, we designed and carried out a suppressor screen for rare mutations that could bypass the *comW* require-

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TABLE 1 Strains and primers used in this study

Strain or primer	Description or sequence (5′–3′)	Source[a] and/or reference	Location
Strains			
CPM7	CP1250 SsbB$^-$::pEVP3::SsbB$^+$; SsbB$^+$; Sm^r Cm^r	13	
CP1250	Rx1 *hex malM511 str-1 bgl-1*; low-α-galactosidase background; Sm^r	1	
CP1344	CP1250 Δ*clpC*::PcTet; Sm^r Tc^r	18	
CP1376	CP1250 *comW*::*kan*; Sm^r Kn^r	9	
CP1500	Rx1 *hex nov-r1 bry-r str-1 ery-r1 ery-r2*; Nv^r Em^r Sm^r	21	
CP1759	CP1250 *comW*::Spc; Spc^r Sm^r	12	
CP2000	CP1250 Δ*cps*; Sm^r	21	
CP2107	CP2000 Δ*cps* SsbB$^-$::pEVP3::SsbB$^+$; SsbB$^+$; Sm^r Cm^r	CP2000 × CPM7 (21)	
CP2136	CP2107 *comA*::Cheshire; Sm^r Cm^r Em^r	35	
CP2137	CP2000 ΔCheshire *comA* mutant; Sm^r Cm^r	35	
CP2451	CP2137 *rpoD*-L363F; Sm^r Cm^r	CP2137 × NYT1 {*rpoD*}	
CP2452	CP2137 *rpoD*-A171V; Sm^r Cm^r	CP2137 × ALT4 {*rpoD*}	
CP2453	CP2137 *rpoD*-R355H; Sm^r Cm^r	CP2137 × FLT4 {*rpoD*}	
CP2454	CP2137 *rpoD*-R316H; Sm^r Cm^r	CP2137 × ILT1 {*rpoD*}	
CP2455	CP2451 Δ*comW*::*kan*; Sm^r Cm^r Kn^r	CP2451 × CP1376 {Δ*comW*::*kan*}	
CP2456	CP2452 Δ*comW*::*kan*; Sm^r Cm^r Kn^r	CP2452 × CP1376 {Δ*comW*::*kan*}	
CP2457	CP2453 Δ*comW*::*kan*; Sm^r Cm^r Kn^r	CP2453 × CP1376 {Δ*comW*::*kan*}	
CP2458	CP2454 Δ*comW*::*kan*; Sm^r Cm^r Kn^r	CP2454 × CP1376 {Δ*comW*::*kan*}	
CP2459	ALT4 *rpoD*-V171; Sm^r Cm^r Kn^r Nv^r Em^r Tc^r	ALT4 × CP2137 {*rpoD*}	
CP2460	FLT4 *rpoD*-H355; Sm^r Cm^r Kn^r Nv^r Em^r Tc^r	FLT4 × CP2137 {*rpoD*}	
CP2461	ILT1 *rpoD*-H316; Sm^r Cm^r Kn^r Nv^r Em^r Tc^r	ILT4 × CP2137 {*rpoD*}	
CP2462	NYT1 *rpoD*-F363; Sm^r Cm^r Kn^r Nv^r Em^r Tc^r	NYT1 × CP2137 {*rpoD*}	
CP2463	CP2137 Δ*comW*::*kan*; Sm^r Cm^r Kn^r	CP2137 × CP1376 {Δ*comW*::*kan*}	
ALT4	CP2463 *rpoD*-A171V; Sm^r Cm^r Nv^r Em^r Tc^r	Spontaneous *rpoD* mutation[b]	
FLT4	CP2463 *rpoD*-R355H; Sm^r Cm^r Nv^r Em^r Tc^r	Spontaneous *rpoD* mutation[b]	
ILT1	CP2463 *rpoD*-R316H; Sm^r Cm^r Nv^r Em^r Tc^r	Spontaneous *rpoD* mutation[b]	
NYT1	CP2463 *rpoD*-L363F; Sm^r Cm^r Nv^r Em^r Tc^r	Spontaneous *rpoD* mutation[b]	
Primers			
DAM497	CAATTGACTATATTAGAGGCGAGACA		*spr0016*
DAM500	TATCAAGCGCATCATTCAAGATAACAG		*purA*
YT18	GCCCTAGAAGAATTGGAACG		*dnaG*
YT20	CAGGGTCGATGACTTCTTCC		*spr0980*
YT30	GACAGGCTTTGAGTCTCTTGATGG		*spr0977*
YT31	CGGACGCTCAAACTTGGCTAATTC		*spr0982*
YT34	CCATTGCCAAACGCTATGTC		*rpoD*
YT36	GTCAACCGCCTTCATCAAGCC		*rpoD*
YT40	TATGGGCTTGATGAAGGC		*rpoD*
YT41	TATGGGCTTGATGAAGGT		*rpoD*
YT42	ATACGCTCACGAGTTACG		*rpoD*
YT43	CGTGAAGAAAATGTTCTGCG		*rpoD*
YT44	CGTGAAGAAAATGTTCTGCA		*rpoD*
YT45	TCACATCTGCCTCGATTG		*cpoA*
YT46	TTGCTACGACTTGGTTGGC		*rpoD*
YT47	TTGCTACGACTTGGTTGGT		*rpoD*
YT48	GTCAATGACCCTGTCCGTATG		*cpoA*
YT49	CAAGTCGTAGCAAACCGC		*rpoD*
YT50	CAAGTCGTAGCAAACCGT		*rpoD*
YT51	CACGGTAAGCACCTGAAAC		*cpoA*
YT76	AGCGCCGACAGGGATTGGGA		*dinG*
YT77	ACATTGGCCTTTTGACGTGCAT		*ezrA*

[a] Braces indicate transfer of the gene only via the PCR amplicon. ×, transformation cross as recipient X donor.
[b] Followed by transformation with *nov-r1*, *ery-r1 ery-r2*, and Δ*clpC*::PcTet donors.

ment and restore the competence lost in Δ*comW* strains. Here, we describe a strategy for recovering rare *comW* bypass mutations and show that they map exclusively to *rpoD*.

MATERIALS AND METHODS

Bacterial strains and culture media. The pneumococcal strains used in the study are listed in Table 1. CP2137, a *cps* Δ*comA* derivative of strain Rx1, was used as the WT standard for transformation assays and as a transformation recipient for the Δ*comW*::*kan* deletion. All strains were cultured in CAT medium, supplemented as needed with 1.5% agar. CAT medium was prepared from 5 g of tryptone (Difco Laboratories), 10 g of enzymatic casein hydrolysate (ICN Nutritional Biochemicals), 1 g of yeast extract (Difco), and 5 g of NaCl in 1 liter of H_2O; sterilized for 40 min at 121°C; and then supplemented to 0.2% glucose and 0.016 M K_2HPO_4

before use. Antibiotics were used at the following concentrations: kanamycin (Kn), 200 μg/ml; novobiocin (Nv), 2.5 μg/ml; erythromycin (Em) 0.075 μg/ml; tetracycline (Tc), 0.25 μg/ml; spectinomycin (Spc), 100 μg/ml; and trimethoprim, 100 μg/ml. CSP1 (22) was obtained from NeoBioSci (Cambridge, MA) as a custom synthetic peptide at 80% purity and stored as sterile 0.025% solution in water at −20°C.

Preparation of donor DNA. The primers used for PCR amplification of donor DNA are listed in Table 1. The *comW*::*kan* deletion fragment was amplified from strain CP1376 using primers DAM497 and DAM500. *rpoD* fragments (5.5 kb) were amplified from strain CP2137 using primers YT30 and YT31. Amplification was performed in 50-μl reaction mixtures with 1 μl Phire HotStart II polymerase (Thermo Scientific) and Phire Reaction Buffer, 10 ng template DNA, 200 μmol/liter of each deoxynucleoside triphosphate (dNTP), and 0.5 μmol/liter of each primer. The amplification conditions were 98°C for 90 s and then 98°C for 15 s, 56°C for 15 s, and 72°C for 105 s for 30 cycles, followed by a 5-min 72°C final extension. The *gyrB* Nv^r marker was prepared as a 7.4-kb amplicon using primers YT76 and YT77 and CP1500 DNA as the template. Amplification was performed as described above, except with 65°C as the annealing temperature. The PCR products were purified using a DNA Clean and Concentrate kit (Zymo Research). Genomic donor DNA was extracted from strains CP1500, CP1344, and CP1759 (Table 1).

Transformation assays. The standard assay for transformation was done essentially as previously described (19). Log-phase culture at an optical density at 550 nm (OD_{550}) of 0.05 at 37°C was incubated with 0.1 μg/ml DNA, 250 ng/ml CSP, 0.5 mM $CaCl_2$, and 0.04% bovine serum albumin for 80 min at 37°C. Portions of the culture were then embedded (in 1.5 ml of CAT mixed with 1.5 ml of CAT agar) in sandwich plates and overlaid with the relevant antibiotic, as previously described (19). After 15 h at 37°C, colonies were counted. The transformation efficiency was expressed as CFU per ml of cells transformed at an OD_{550} of 0.05. To determine the transformation efficiency relative to the WT, the transformant yield of a strain was divided by that for a parallel WT culture. In the WT, typical yields for the PCR-amplified Nv^r donor were 50% to 90% of cells transformed, while typical yields for the genomic DNA preparations were ~2%, ~1%, and 0.1 to 0.3% for Em^r, Tc^r, and Spc^r markers, respectively.

Estimation of spontaneous mutation frequency. An indication of the number of spontaneous mutations present in a mutant library was obtained by determining the number of trimethoprim-resistant mutants, which arise by inactivation of any of several proteins, including thymidine synthetase and the Ami transporters. Mutants insensitive to 100 to 200 μg/ml trimethoprim occurred at a frequency of $\sim 10^{-4}$.

Transformation and recovery of enrichment libraries. Mutant libraries were prepared by serial dilutions of cells from a single isolated colony of CP2463 for 35 doublings in culture volumes of 10 ml. To obtain an enriched suppressor mutant library, 10^8 CFU in 1 ml CAT were exposed to CSP and DNA as described for the standard transformation assay. The entire culture was spread onto 20 100-mm selective CAT agar plates. After 15 h at 37°C in 5% CO_2, 2×10^4 of the resulting transformant colonies were resuspended in 10 ml CAT. During outgrowth at 37°C for several hours, viable cells increased from $\sim 10^5$ to 10^8 CFU/ml. This pool of transformant clones was stored at −80°C with 10% glycerol. The recovered transformant library pool is estimated to contain 20,000 transformant clones, each at 5,000 cells/ml.

Whole-genome sequencing (WGS) and analysis. Genomic DNA for sequencing was prepared from 10^9 CFU by 0.2% Triton lysis and purified using a Genomic DNA Clean and Concentrate kit (Zymo Research). For sequencing on the Ion Torrent Proton sequencer, genomic DNA extracts were sheared to an average fragment size of 200 bp using a Covaris S220 focused ultrasonicator and purified using AMPure beads (Beckman-Coulter, Brea, CA). Library preparation followed the Wafergen Prep PGM 200 DNA Library protocol, using an Apollo 324 system (Wafergen, Fremont, CA) according to the manufacturer's instructions. DNA libraries containing Ion Xpress Barcode Adapters (Life Technologies, Grand Island, NY) were quantified in a Qubit 2.0 fluorometer (Life Technologies) and pooled in approximately equimolar ratio. The pool was quantified using the Library Quantification kit for Ion Torrent (KAPA Biosystems, Woburn, MA) and diluted to 26 nM prior to emulsion PCR, which was performed on the OneTouch2 instrument (Life Technologies) according to the manufacturer's instructions. Subsequently, the emulsion was broken, and Ion sphere particles (ISPs) containing amplified DNA fragments were recovered using the OneTouchES instrument (Life Technologies). The ISPs were loaded onto a Proton I chip and analyzed on the Ion Torrent Proton sequencer using 200-bp chemistry (V2) according to the manufacturer's instructions. Sequence data were demultiplexed on the Proton server and exported as FASTQ files. Using the CLC genomics workbench (CLC Bio), sequencing reads from each strain were mapped to the reference genome sequence of strain R6 (23), a laboratory ancestor of Rx1 (24). Variants that indicated a single nucleotide polymorphisms (SNP) in any of the strains relative to the reference were compiled (see Data set S1 in the supplemental material) and filtered as described in Results (see Table S1 in the supplemental material).

Targeted gene sequencing at the *rpoD* locus. To sequence DNA near the *rpoD* gene, a 5.5-kb amplicon was prepared using primers YT30 and YT31 as described above, followed by sequencing reads using primers YT18, YT20, YT34, and YT36 at the University of Illinois at Chicago (UIC) Research Resources Center (RRC) sequencing facility, providing >3-fold coverage of 100% of the *rpoD* gene.

Allele exchange crosses. To avoid possible disruption of the *dnaG-rpoD-spr0980* operon, candidate suppressor alleles were transferred directly by transformation, without any linked selective marker gene, by taking advantage of the high replacement frequency achieved during natural genetic transformation with large, pure donor gene fragments. To replace one *rpoD* allele with another, a 5.5-kb amplicon encompassing *rpoD* and flanking genes, prepared by PCR from cells from a donor strain template with primers YT30 and YT31, as described above, was used as a donor at 100 ng/ml to transform a competent culture of the recipient strain. Single colonies were scanned for the replacement allele by SNP determination as described below. Overall, the frequencies of allele replacement under these conditions were 60% for the WT and 12% for suppressed *comW* mutants. A purified subclone of each positive colony, verified by sequencing the entire *rpoD* gene, was retained for further analysis.

SNP determination by mismatched-primer assay. *rpoD* alleles were distinguished by a mismatched-primer PCR assay. Primers were designed to match ~20 bases, including each SNP as the 3′-terminal base of the primer: YT41, YT44, YT47, and YT50. Identical primers, except for the 3′-terminal base, which matched the WT sequence, were also created: YT40, YT43, YT46, and YT49. They were paired with a second primer, YT42, YT45, YT48, or YT51, to amplify a 500-bp diagnostic fragment. Thus, primer set YT40, YT41, and YT42 was used to detect the base change corresponding to the A171V amino acid substitution. Using YT40 or YT41 as the forward primer and YT42 is the reverse primer, two separate reactions were performed. The two reaction products were compared on a gel for the presence or absence of a band. The same was done with primer sets YT43, YT44, and YT45 for R316H; YT46, YT47, and YT48 for R355H; and YT49, YT50, and YT51 for L363F. Detection of a product with the WT primers indicated a WT *rpoD* sequence, while a product with only the primers matching the mutant allele indicated a mutant *rpoD* allele. Amplification was performed in 10-μl reaction mixtures, with 0.2 μl Phire HotStart II polymerase (Thermo Scientific) and Phire Reaction Buffer, 1 ng template DNA, 200 μmol/liter of each dNTP, and 0.5 μmol/liter of each primer. The amplification conditions were 98°C for 90 s and then 98°C for 15 s, 69°C for 15 s, and 72°C for 15 s for 20 cycles, followed by a 5-min 72°C final extension.

RESULTS

Rare Δ*comW* suppressors are recovered by serial enrichment. The expression of genes regulated by an alternative sigma factor typically depends on numerous additional factors affecting the entire life cycle of the sigma factor itself, including its synthesis, its

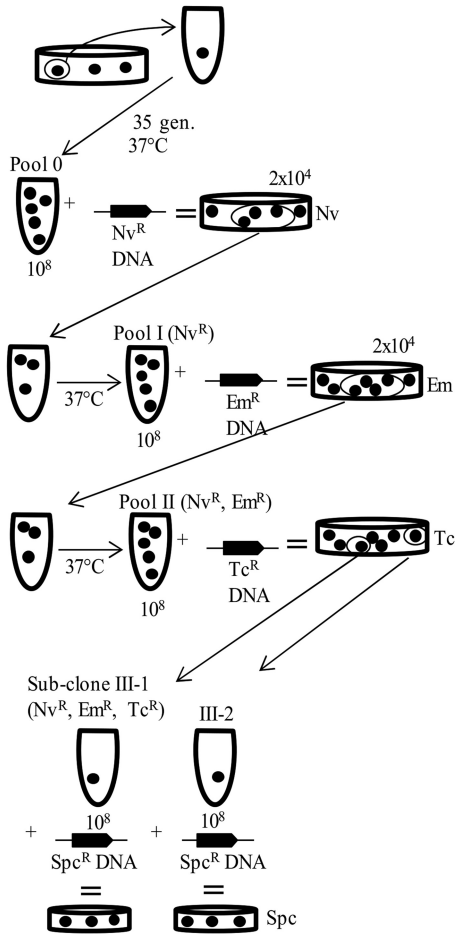

FIG 1 Strategy for enrichment of *comW* bypass mutants. A single colony was picked and grown for 35 generations to create pool 0, a library of potential suppressor mutations. Then, 10^8 cells of pool 0 were transformed with Nv^r DNA and plated, and 2×10^4 Nv^r transformant colonies were collected to create pool I. Next, 10^8 cells of pool I were transformed with Em^r DNA, and 2×10^4 Nv^r Em^r transformant colonies were collected to create pool II. After 10^8 cells of pool II were transformed with Tc^r DNA, individual Nv^r Em^r Tc^r transformant colonies were picked and transformed with Spc^r DNA to determine the transformation efficiency. The dots in tubes represent cells; the dots on plates represent colonies.

TABLE 2 Recovery of suppressor mutants through serial transformational enrichment

Culture[a]	Transformation efficiency[b]		Relative efficiency[e]
	WT[c]	Mutant[d]	
Spontaneous-mutation library (pool 0)	5×10^7	2×10^4	0.0004
Collected Nv^r library (pool I)	5×10^5	1×10^2	0.0002
Collected Nv^r Em^r library (pool II)	5×10^5	5×10^2	0.001
Nv^r Em^r Tc^r clone 1	1×10^5	3×10^2	0.003
Nv^r Em^r Tc^r clone 2	1×10^5	9×10^1	0.0009
Nv^r Em^r Tc^r clone 3	1×10^5	4×10^3	0.04
Nv^r Em^r Tc^r clone 4	1×10^5	1×10^4	0.1
Nv^r Em^r Tc^r clone 5	1×10^5	1×10^4	0.1
Nv^r Em^r Tc^r clone 6	1×10^5	2×10^4	0.2

[a] Pool or subclone in enrichment series PRT.
[b] Transformation efficiency, number of drug-resistant CFU/ml, determined in quadruplicate. A PCR marker was used for pool 0, and genomic markers were used for pools I and II and clones 1 to 6. SD values were below 20%.
[c] WT control.
[d] Mutant pool or clone.
[e] Relative efficiency, transformation efficiency of the strain or pool relative to that of the WT.

availability, its stability, its success in competing for occupancy of limiting core polymerase, and interaction of the resulting holoenzyme with specific promoter sites. As existing data indicate that ComW might affect various levels in the life cycle of σ^X, including synthesis, stability, and activity, but no molecular mechanism has yet been identified, we wished to obtain an unbiased genetic indication of the most important site of action of ComW by collecting and mapping suppressor mutations that could partially bypass the requirement for *comW* in competence development.

Although the severe transformation deficiency of *comW* mutants (10,000-fold reduced) offers the possibility of strong enrichment for such suppressors in a single step of transformation, a preliminary screen of a transposon library (provided by P. Burghout, Nijmegen, The Netherlands) did not yield any such suppressors; because transposon insertions produce predominantly null mutations, this may indicate that important players might be essential for viability (data not shown). To allow recovery of suppressors that were not null mutations, we sought conditions that would provide a sufficiently rich array of base substitutions to allow recovery of more subtle bypass mutations but not such a high density of base changes as to make mapping by WGS cumbersome. Because strain Rx1 carries a mutation inactivating the HexAB mismatch repair system, which creates a mild mutator phenotype (24), we conducted a pilot enrichment in the Rx1 derivative CP2463, in which *comW* was replaced by a Kn^r cassette and a *comA* deletion made development of competence absolutely dependent on exogenous CSP.

A single *comW* mutant colony of strain CP2463 was grown for ~35 doublings to create a library of mixed, potentially mutant subclones (Fig. 1). The total frequency of trimethoprim-resistant mutants in a similar culture was estimated as 0.01% (data not shown), indicating the presence of numerous spontaneous base pair changes in the pool. Subjecting this library to transformation

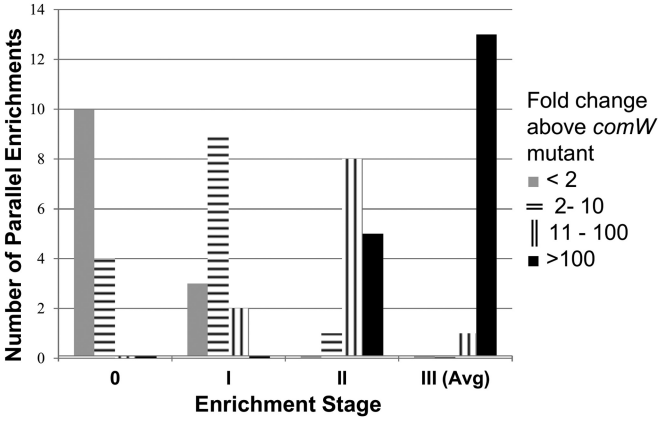

FIG 2 Serial enrichment of suppressor mutants from 14 independent libraries. Shown are counts of pools with transformation efficiencies in four ranges, for the initial mutant library (stage 0) and 3 successive cycles of enrichment. Values for enrichment stage III reflect averages (Avg) of subclone efficiencies (Table 3).

TABLE 3 σ^A substitutions in bypass mutants

Independent enrichment[a]	Individual[b]	Relative efficiency[c]	σ^A substitution[d]
ALT	5	0.002	R297C
	2	0.007	R316C
	1	0.01	P287S
	3	0.02	A171V
	4	0.05	**A171V**
DET	1	0.0003	−
	3	0.006	A171V
	4	0.009	−
	5	0.05	**A171V**
FLT	3	0.004	R355H
	5	0.006	R355H
	1	0.03	R316H
	4	0.08	**R355H**
GAT	5	0.00008	−
	4	0.02	R316H
	2	0.06	R316H
	3	0.08	A171V
	1	0.09	**R316H**
ILT	3	0.0004	−
	4	0.1	R316H
	2	0.26	R316H
	1	0.3	R316H
	5	0.31	**R316H**
MAT	2	0.002	−
	1	0.003	−
	4	0.003	Y290C
	3	0.01	R316H
	5	0.02	**A171V**
MTT	4	0.008	R316H
	3	0.03	A171T
	2	0.06	A171V
	1	0.07	R316H
	5	0.07	**R316H**
NCT	1	0.001	−
	3	0.02	A171V
	5	0.02	A171V
	2	0.07	**A171V**
	4	0.07	A171V
NVT	2	0.003	V314A
	4	0.02	A171V
	5	0.02	A171V
	3	0.07	V314A
	1	0.1	**R316H**
NYT	5	0.00008	−
	4	0.008	L363F
	1	0.18	**L363F**
	3	0.28	R316H
	2	0.41	**R316H**
OHT	1	0.05	**R355H**
PRT	2	0.0009	−
	1	0.003	−
	3	0.04	R355H
	4	0.1	R316H
	5	0.1	R316H
	6	0.2	R316H
SCT	5	0.08	R316H
	4	0.1	**R316H**
	2	0.13	R316H
TXT	2	0.06	R316H
	5	0.1	R316H
	4	0.11	**R316H**
WT		1	WT
Δ*comW*		0.0001	WT

[a] Name of enrichment series.
[b] Tc^r clone number.
[c] Ratio of mutant transformation efficiency to that of the WT. The SD values of quadruplicate measurements were below 20%.
[d] Boldface, mutation identified by WGS; −, isolate not sequenced.

could preferentially retrieve mutations that suppress the loss of transformation caused by ComW deficiency, but as the transformation efficiency of a Δ*comW* mutant is nonzero (10^{-4}), transformants of rare-suppressor-bearing mutants would not be expected to dominate the pool of transformants obtained from a single round of transformation. However, we anticipated that the enrichment achieved in this pool would be compounded by a second round of transformation of the entire transformant pool and that suppressor mutants might predominate after further repetition of the enrichment.

To maximize recovery of rare suppressors in the first cycle of enrichment, a pure 7.4-kb Nv^r DNA amplicon, which routinely transforms the WT with 80 to 90% efficiency, was chosen to transform a portion of the mutant library (100 million cells). Transformant colonies (2×10^4) were collected as a pool of Nv^r cells and outgrown to produce a working pool stock. This pool was not rich in suppressors (Table 2); indeed, the recovered Nv^r library did not transform detectably more than the Δ*comW* background rate of 10^{-4} compared to the WT strain. The pool of Nv^r transformants was next transformed with a genomic Em^r donor DNA. Among 20 resulting Em^r transformants, none transformed better than the Δ*comW* mutant (data not shown). Since suppressors were thus clearly not dominant among the resulting Nv^r Em^r transformants, a second enrichment library was collected as a pool of 20,000 of the Nv^r Em^r transformants (Fig. 1) and subjected to a third cycle of transformation using genomic Tc^r donor DNA (Fig. 1). Among six resulting Nv^r Em^r Tc^r transformants, four transformed at a much higher frequency (400- to 2,000-fold) than the *comW* mutant parent (Table 2). This indicates that suppressors with increased transformation efficiencies dominated the resulting Nv^r Em^r Tc^r transformant collection and established that three cycles of enrichment by this procedure could suffice to recover rare stable *comW* bypass mutants.

To obtain additional independent bypass mutants, this compound enrichment process was repeated in parallel for 13 addi-

TABLE 4 Filtering of SNPs detected by WGS in 14 suppressor strains

Stage of analysis	Total no. of SNPs in 14 strains	No. of SNPs per strain
All SNPs detected vs. R6[a]	9,000	640
High-quality sequence coverage[b]	7,500	540
Changes in ORFs[c]	6,600	470
Nonsynonymous	3,900	280
Absent from parent[d]	132	9
Outside introduced markers[e]	68	5
In *rpoD*	14	1

[a] Total SNPs after alignment to accession no. NC_003098.1.
[b] SNPs remaining after removal of SNPs with poor sequence coverage.
[c] Open reading frames (ORFs) as annotated in accession number NC_003098.1.
[d] SNPs absent from the Δ*comW* parent.
[e] SNPs not introduced by Nv^r, Em^r, or Tc^r transformation.

tional separate single-colony subclones of CP2463, following the same series of successive enrichment cycles. After the first enrichments, 9 of the 14 Nv^r pools transformed at slightly elevated rates, while two transformed 10 times as well as the *comW* parent, indicating that suppressor mutants were present in the pools, but at a low frequency (Fig. 2). After the second series of enrichment cycles, 5 of the 14 Nv^r Em^r pools transformed at rates at least 100-fold above that of the *comW* parent. After the third enrichment, among five Nv^r Em^r Tc^r transformant subclones tested from each enrichment, at least one transformed at a rate at least 20-fold higher than that of the Δ*comW* parent (Fig. 2 and Table 3), indicating that suppressor mutants dominated in all 14 cases.

We conclude that rare, spontaneously arising suppressors of the *comW* transformation deficiency can be recovered repeatedly by three cycles of compounded enrichments.

All suppressor mutants contain an SNP in *rpoD*. Since com-

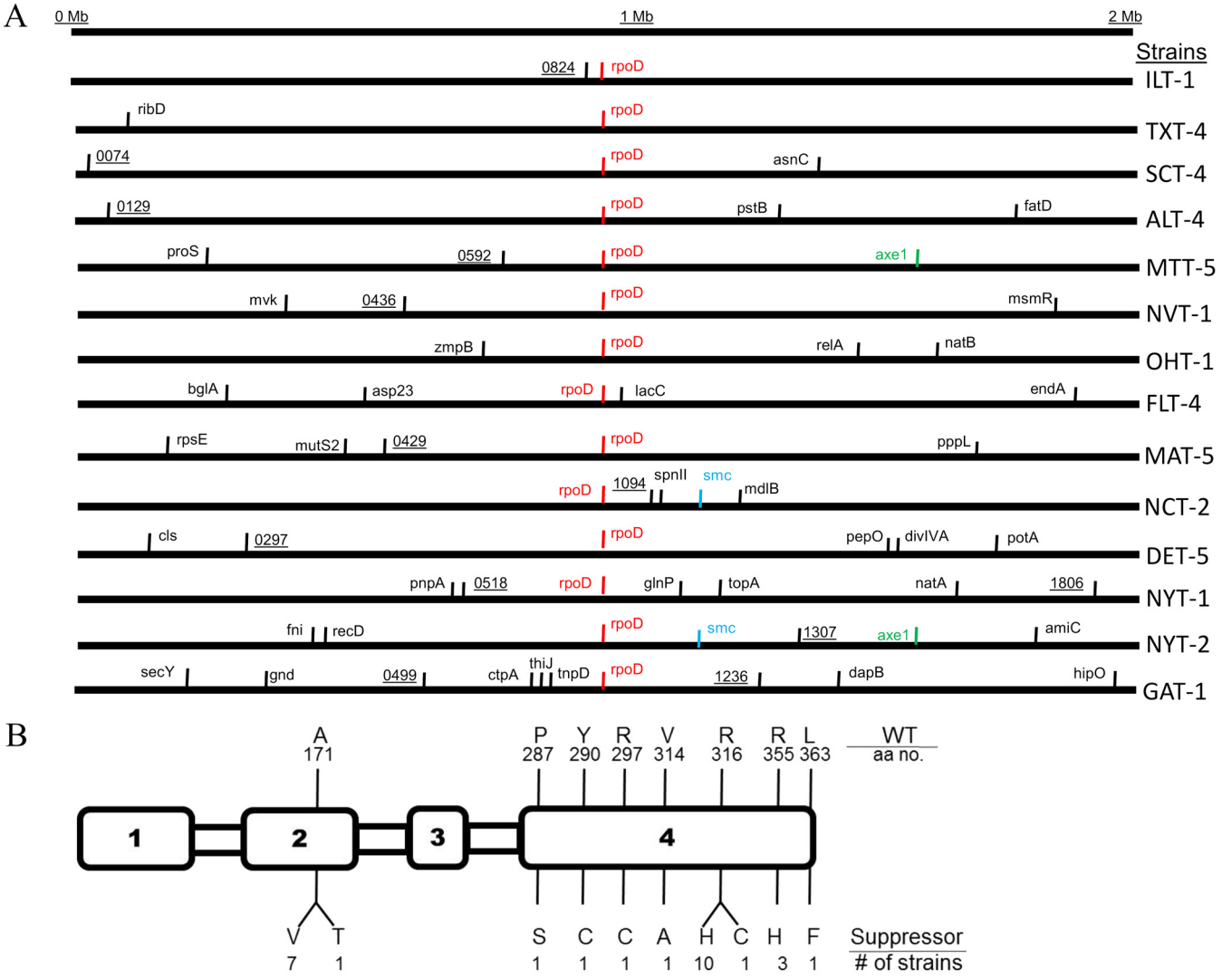

FIG 3 Locations of 68 amino acid changes identified by whole-genome and *rpoD*-targeted sequencing. (A) Mapping of base changes identified by WGS in 14 independent *comW* bypass mutants. Nonsynonymous substitutions are organized by relative genome positions, and gene designations are as annotated in accession number NC_003098.1. Black, unique gene hit; color, 2 or more hits in the same gene; black horizontal line, entire *S. pneumoniae* genome. The names of the bypass strains sequenced by WGS are on the right. (B) Map of predicted amino acid residue changes in RpoD (σ^A) among 27 suppressor mutants (Table 3). Boxes, four conserved regions of σ^A, 369 amino acids (aa), as assigned by Vassylyev et al. (32). The WT residues and positions are shown above the protein, and the suppressor residues and numbers of cases are shown below.

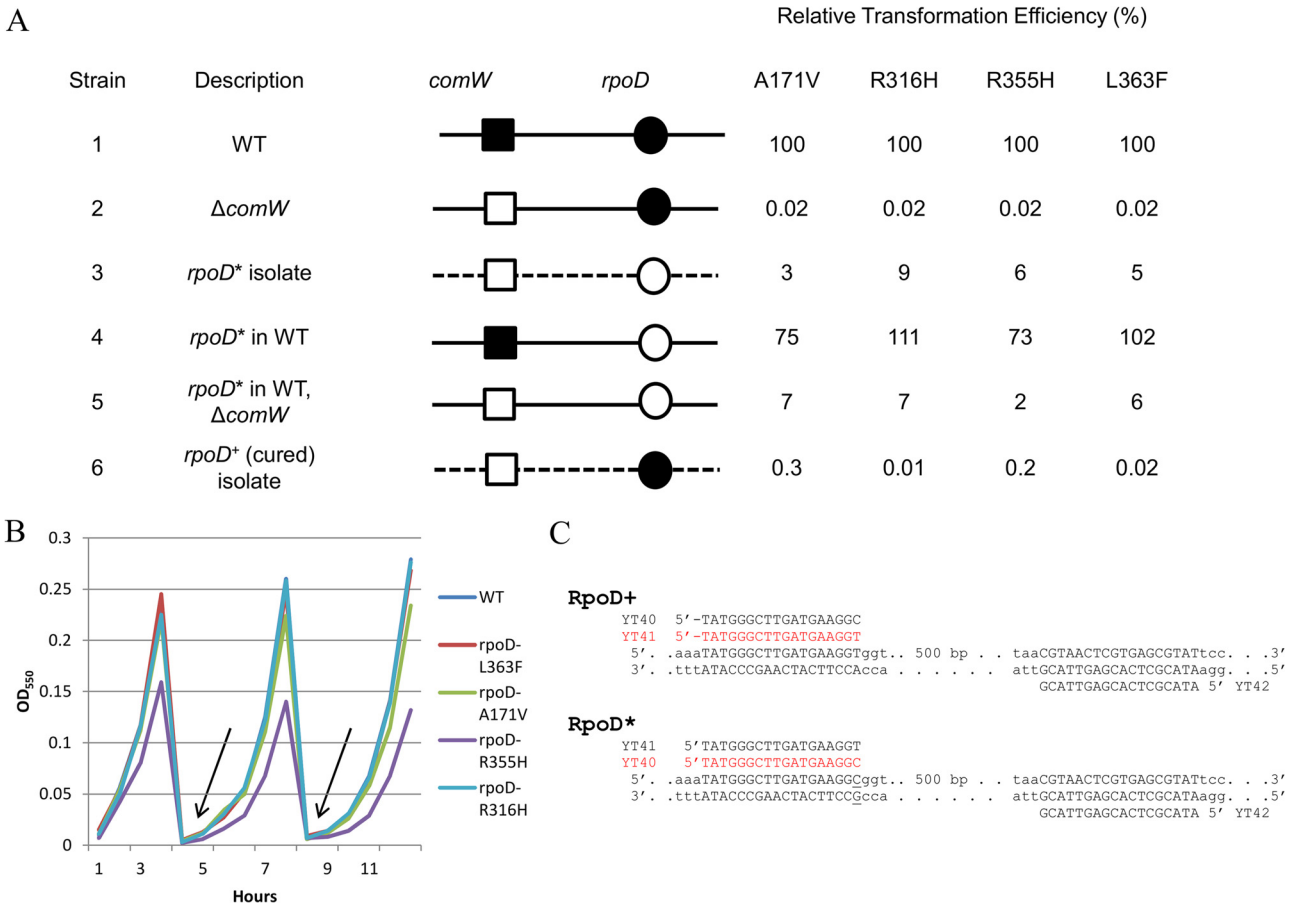

FIG 4 Linkage between *rpoD** mutations and the *comW* bypass phenotype. (A) Backcross analysis of the bypass phenotypes of four *rpoD** isolates. Shown is a comparison of the transformation efficiency of the suppressor isolate (line 3) to those of isolates with *rpoD** in a WT background (line 4) and with *rpoD** in a transformant Δ*comW* WT background (line 5) and a suppressor isolate cured of the *rpoD** mutation (line 6). Solid lines, WT genome; dashed lines, genome of isolate recovered from enrichment; ■, *comW*$^+$; □, Δ*comW*; ●, *rpoD*$^+$; ○, *rpoD** (the bypass mutant residues are indicated at the tops of the data columns). For mutations A171V, R316H, R355H, and L363F, the suppressor isolates used were ALT4, ILT1, FLT4, and NYT1, respectively; the strains with backcrossed *rpoD** alleles in a Δ*comW* WT background were CP2456, CP2458, CP2457, and CP2455; and the *rpoD*$^+$ cured strains were CP2459, CP2461, CP2460, and CP2462. Standard deviation (SD) values were below 20%. (B) Growth of strains CP2451 to -54 containing *rpoD** mutations over 17 generations. The arrows indicate 1:100 dilutions of the exponentially growing culture in fresh medium. (C) Allele-specific alternative primer pairs used for mismatch PCR genotyping of segregants after transformation with 5.5-kb donor *rpoD* amplicons. The SNP in the A171V mutant is underlined. The productive allele-specific primer for each allele is shown in red.

petence was substantially restored in each of at least 14 independently derived Δ*comW* mutants, we sought to learn whether suppression was caused by single-gene mutations and to identify mutations that might be linked to the suppressor phenotype. To identify candidate unmarked mutations, WGS was carried out on a total of 16 strains, including 14 suppressor mutants, the Δ*comW* parent (CP2463), and the corresponding *comW*$^+$ wild type (CP2137). The sequencing reads were aligned to an annotated reference sequence of *S. pneumoniae* strain R6 (23), which is derived, like Rx1, from the clinical isolate D39 (25). Approximately 9,000 SNPs were identified in total (~600 per sequenced strain) (Table 4; see Data set S1 in the supplemental material). After removing from consideration SNPs in regions with less than 40-fold coverage, ~6,600 SNPs mapped to coding regions as identified in the R6 genome annotation. Of these, ~2,700 cause synonymous amino acid changes. Among the remaining 3,900 SNPs, 3,768 reflected divergence of the Rx1 and R6 lineages (24), i.e., differed from the R6 sequence but were present in all of our sequenced strains (14 suppressors, the WT, and the Δ*comW* parent). Among the remaining 132 SNPs, 64 were mapped to donor drug markers introduced during enrichment cycles. The remaining 68 SNPs of interest were distributed among 53 unique genes among the sequenced suppressor strains, ranging from 2 to 10 SNPs per strain. However, only the gene *rpoD* contained an SNP in each of the 14 sequenced suppressor strains (Table 4; see Table S1 in the supplemental material). As the remaining 54 SNPs were scattered among 52 other genes (Fig. 3A), we hypothesized that the single gene hit in every suppressor strain, *rpoD*, was the site of the effective bypass mutations.

Using Sanger sequencing of PCR products, we confirmed the presence of each *rpoD* mutation in the 14 suppressor strains used for WGS and the absence of any *rpoD* mutation from the WT, the Δ*comW* parent, and the Nv^r, Tc^r, Spc^r, and Em^r donor strains, indicating that the mutations arose during the mutagenesis and enrichment cycles. When *rpoD* was similarly sequenced in the remaining 37 suppressor mutants identified among cycle 3 Nv^r Em^r Tc^r transfor-

```
T. aquaticus      AKKYTGRGLSFLDLIQEGNQGLIRAVEKFEYKRRFKFSTYATWWIRQAINRAIADQARTI    273
E. coli           AKKYTNRGLQFLDLIQEGNIGLMKAVDKFEYRRGYKFSTYATWWIRQAITRSIADQARTI    450
S. aureus         AKRYVGRGMLFLDLIQEGNMGLIKAVEKFDFNKGFKFSTYATWWIRQAITRAIADQARTI    206
S. pneumoniae     AKRYVGRGMQFLDLIQEGNMGLMKAVDKFDYSKGFKFSTYATWWIRQAITRAIADQARTI    206
S. mutans         AKRYVGRGMQFLDLIQEGNMGLMKAVDKFDYSKGFKFSTYATWWIRQAITRAIADQARTI    208
                  **:*. **: ********* **::**:**:: : :**************.*:********

T. aquaticus      RIPVHMVETINKLSRTARQLQQELGREPSYEEIAEAMGPGWDAKRVEETLKIAQEPVSLE    333
E. coli           RIPVHMIETINKLNRISRQMLQEMGREPTPEELAERMLMP--EDKIRKVLKIAKEPISME    508
S. aureus         RIPVHMVETINKLIRVQRQLLQDLGRDPAPEEIGEEMDLP--AEKVREILKIAQEPVSLE    264
S. pneumoniae     RIPVHMVETINKLVREQRNLLQELGQDPTPEQIAERMDMT--PDKVREILKIAQEPVSLE    264
S. mutans         RIPVHMVETINKLVREQRNLLQELGQDPTPEQIAERMDMT--PDKVREILKIAQEPVSLE    266
                  ******:****** *  *:: *::*::*: *::.* *      .::.: ****:**:*:*

T. aquaticus      TPIGDEKDSFYGDFIPDENLPSPVEAAAQSLLSEELEKALSKLSEREAMVLKLRKGLIDG    393
E. coli           TPIGDDEDSHLGDFIEDTTLELPLDSATTESLRAATHDVLAGLTAREAKVLRMRFGIDMN    568
S. aureus         TPIGEEDDSHLGDFIEDQEAQSPSDHAAYELLKEQLEDVLDTLTDREENVLRLRFGLDDG    324
S. pneumoniae     TPIGEEDDSHLGDFIEDEVIENPVDYTTRIVLREQLDEILDTLTDREENVLRLRFGLDDG    324
S. mutans         TPIGEEDDSHLGDFIEDEVIENPVDYTTRVVLREQLDEVLDTLTDREENVLRLRFGLDDG    326
                  ****::.**. **** *     * : ::   *    .. *  *: **  **::* *:

T. aquaticus      REHTLEEVGAYFGVTRERIRQIENKALRKLKYHESRTRKLRDFLE-      438
E. coli           TDYTLEEVGKQFDVTRERIRQIEAKALRKLR-HPSRSEVLRSFLDD      613
S. aureus         RTRTLEEVGKVFGVTRERIRQIEAKALRKLR-HPSRSKRLKDFMD-      368
S. pneumoniae     KMRTLEDVGKVFNVTRERIRQIEAKALRKLR-QPSRSKPLRDFIED      369
S. mutans         KMRTLEDVGKVFDVTRERIRQIEAKALRKLR-HPSRSKQLRDFVED      371
                     ***:**  * ********** ******: : **:. *:.*::
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FIG 5 Alignment of σ^A homologs. *S. pneumoniae* σ^A amino acid residues 147 to 369 were aligned with σ^A sequences from accession numbers Q9EZJ8.1 (*T. aquaticus*), YP_491259.1 (*E. coli*), EIA15345.1 (*S. aureus*), NP_721232.1 (*S. pneumoniae*), and NP_358573.1 (*S. mutans*), using Clustal Omega with default parameters (33). Asterisks, identical residues; colons, conserved residues; periods, semiconserved residues; red, residues in *S. pneumoniae* σ^A replaced by bypass mutations.

mants (Table 3), several more independent instances of the four *rpoD* mutations identified by WGS were revealed, as well as six additional *rpoD* nucleotide changes, as shown in Fig. 3B.

Altogether, the mapping and sequence data supported an inference that bypass of the *comW* requirement is available solely or principally by specific modification of σ^A.

Four mutant *rpoD* alleles substantially rescue transformation in Δ*comW* strains and account for most or all of the suppression phenotype. To determine directly whether the mutations identified in *rpoD* could bypass the loss of competence in *comW* mutants, two sets of allele replacement crosses were done for four representative *rpoD* suppressor alleles, including the three that had been recovered most frequently. Each selected *rpoD* allele was transferred to a WT background, followed by deletion of *comW* to allow detection of possible suppression. Conversely, the *rpoD** allele (where the asterisk indicates any of the four mutant *rpoD* alleles) in each of the four corresponding suppressor mutants was replaced by the wild-type sequence (Fig. 4A). Since the mutations were not linked to a selective marker, we used a single-nucleotide primer mismatch PCR assay (Fig. 4C) to distinguish *rpoD* transformants from untransformed progeny. In all four cases, the backcross of mutant *rpoD* alleles was sufficient to increase transformation in a *comW* background to the level observed in the original suppressor mutant (Fig. 4A). In each case, introduction of the putative suppressor allele in the WT background did not itself detectably affect the transformation efficiency or growth rate (Fig. 4A and B). However, after the removal of *comW*, each *rpoD** *comW* double mutant strongly suppressed the transformation deficiency typical of *comW* mutants. Thus, suppression of the *comW* transformation defect was indeed linked to the mutant *rpoD** alleles and not to any other mutations that may have accumulated during enrichment of suppressor mutants.

To test the linkage of suppression to the mutant alleles of *rpoD* further, we cured each of the four suppressor mutant types of its *rpoD** SNP by replacement with the WT *rpoD* (Fig. 4A). For each mutation, one suppressor isolate was selected at random for the cross, although three of the mutations arose more than once. On curing of their *rpoD* mutations, the four strains lost their suppression phenotype, two completely and two partially (Fig. 4A). In the former cases, this indicates that suppression is entirely explained by the mutations in *rpoD*. In the latter cases, it appears to be possible that an additional mutation is present in the recovered suppressor mutants that accounts for a modest level of suppression (~10-fold higher than that of the *comW* mutant). Nonetheless, as the corresponding backcross strains containing only the mutant allele of *rpoD* transformed at the level of the original suppressor mutant, it is clear that in both cases the mutant *rpoD* allele was primarily responsible for the *comW* bypass phenotype.

We conclude that each of the four substitutions in σ^A, A171V, R316H, R355H, and L363F, is individually both necessary and sufficient for *comW* bypass in the suppressor mutants recovered through repeated transformational enrichment.

DISCUSSION

To seek clues to the critical function of ComW during competence development, rare suppressors were retrieved from a library of spontaneous mutants prepared in a *comW* mutant background. This strategy provided a minimally biased way to identify possible ComW interaction partners, especially by allowing the examination of essential genes. Whole-genome sequencing and linkage analysis identified 10 different spontaneous mutations that substantially increased transformation efficiency in the ComW-deficient background. Remarkably, all 10 suppressors mapped to *rpoD*, which encodes the essential primary sigma factor subunit of RNA polymerase. This immediately implicates the housekeeping sigma factor in the function of ComW during competence development in *S. pneumoniae* and may explain why a previous approach using a mariner transposon was not successful at identifying *comW* suppressors.

Although there is no currently available structure for the *S. pneumoniae* RNA polymerase holoenzyme, the strong conserva-

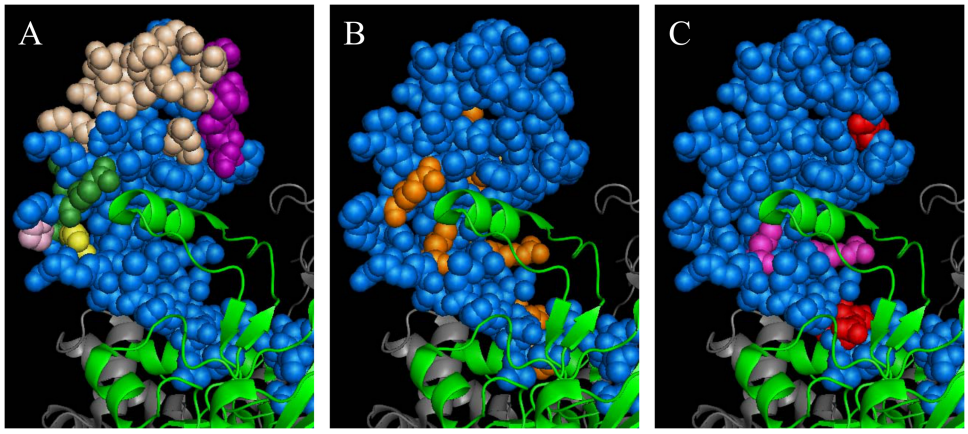

FIG 6 Locations of DNA-contacting and protein interaction residues, *comW* bypass residues, and bypass and affinity-affecting residues in region 4 of σ^A in a holoenzyme. Shown is the crystal structure of a holoenzyme from *E. coli* (34), Protein Data Bank (PDB) ID 4LJZ. σ^{70}, space-filling blue; β-subunit, green ribbon; β′, gray ribbon. (A) Residues contacting DNA or regulatory proteins in region 4. Beige, DNA-binding residues; dark green, CAP-interacting residues; pink, FNR-interacting residues; yellow, λCI-interacting residues; purple, PhoB-interacting residues (all according to Campbell et al. [26]). (B) *comW* suppressor residues in region 4. Orange, residues corresponding to *comW* bypass mutations identified in this study. (C) Bypass and affinity residues from *E. coli* and *T. thermophilus* in region 4. Red, residues corresponding to bypass mutations that facilitate the activity of an alternative sigma factor according to Laurie et al. (29); magenta, mutations that reduce σ^A affinity for RNA according to Dove et al. (27) and Nickels et al. (28).

tion of the sequence and structure of bacterial RpoD proteins suggests it could be informative to inspect the structure of σ^{70} in the *Escherichia coli* holoenzyme for clues to the nature of the pneumococcal *comW* bypass mechanism. To identify homologous residues in *E. coli* and *Thermus aquaticus* σ^{70} that correspond to those affected in the pneumococcal bypass mutants, we aligned regions 2 to 4 of RpoD proteins from several species (Fig. 5). To make comparisons, we used *E. coli*, *T. aquaticus*, and *Staphylococcus aureus* sequences, for which there are available crystal structures, and the *Streptococcus mutans* sequence, because it is a *Streptococcus* species that is naturally transformable but does not have ComW. Region 4 of σ^A, the site of most of the bypass substitutions, forms a protrusion rooted by region 3 in the cleft between the β and β′ subunits (Fig. 6). In the shape of a slightly cupped left hand, it exposes several surfaces with known functions in the initiation of transcription. The base knuckles and thumb are targeted by many transcriptional activator proteins, such as CAP and the λCI repressor, while residues of the little finger interact with PhoB (Fig. 6A) (26). The palm forms a hydrophobic surface that cradles the β flap tip, while the wrist also contacts core residues in the β and β′ subunits. The *comW* bypass substitutions identified here map exclusively to the palm and wrist, surfaces thought to interact with core subunits but not with either promoter DNA or known DNA-binding regulators (Fig. 6B). However, the latter surfaces do contain residues that are important in *E. coli* and *T. aquaticus* for core affinity (27, 28), as well as residues altered by DksA/ppGpp bypass mutations that facilitate activity of the alternative sigma factor σ^N (Fig. 6C) (29). Colocation of residues altered in *comW* bypass mutants with DksA bypass residues and core affinity residues suggests that ComW may serve in some way to modulate σ^A/σ^X competition in favor of σ^X and that weakening σ^A affinity for core renders this ComW activity less critical for competence development. If σ^A were to have a reduced affinity for core, it is possible that this would manifest in observable changes in growth. We did not observe such changes under standard growth conditions (Fig. 4B), and the literature regarding the residues affecting ppGpp bypass (29) and affinity for core (27, 28) lacks any observations on corresponding changes in the growth rate. However, there are some mutations in *rpoD* that cause growth defects under certain conditions, such as high temperatures (30) and exposure to ppGpp (31), which we have not yet examined. The alanine residue affected by the remaining two *comW* bypass mutations (*S. pneumoniae* A171 or *E. coli* A415) is buried within the three-dimensional (3D) structure of region 2, where it forms part of the interface between two successive alpha-helices (E399-D417 and K425-Q445). We speculate that disruption of this packing may also weaken the interaction between σ^A and core.

Despite the predominant location of *comW* bypass mutations in areas of σ^A that interact with core subunits, neither the mechanism of action of ComW nor the mechanism bypassing the *comW* require-

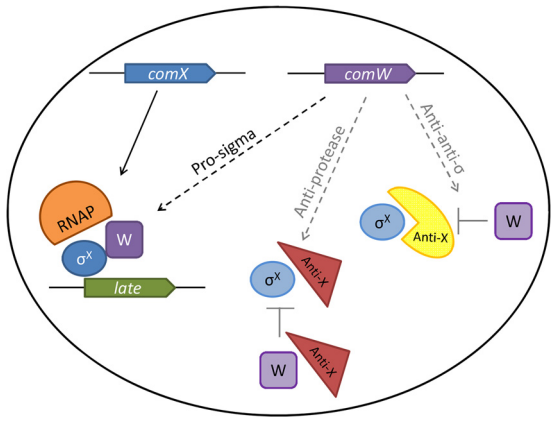

FIG 7 Model of potential ComW functions. Possible functions of ComW as a prosigma, anti-protease-adapter or as an anti-anti-σ. The prosigma function promoting competition with σ^A is in boldface because it is consistent with recovery of bypass mutations exclusively in *rpoD*. Anti-protease and anti-anti-σ functions are lighter because no bypass mutations were in protease subunits/adapters or in other proteins orthologous to anti-σ factors. Dashed lines, possible functions; pentagons, genes; orange half-circle, core RNA polymerase; blue circles, σ^X; purple squares, ComW; yellow, anti-σ^X; red triangles, protease.

ment is revealed directly by these data. They do not even reveal which of the *comW* phenotypes are relieved in the bypass mutants. The location of affected residues in bypass mutants simply points to σ^A/σ^X competition (Fig. 7), which could be impacted by mechanisms affecting the level of σ^X or by those affecting more directly its interaction with core polymerase. However, it can be thought to "tip the balance" among known mechanisms affecting alternative sigma factor activity if the absence of other suppressor sites is considered. For example, activity of ComW either as an anti-anti-sigma or as an anti-protease-adapter would imply that these partners could be sites for additional effective suppressor mutations. The absence of such sites from our collection of suppressors suggests they are not important targets of ComW activity. In contrast, exclusive targeting of this part of σ^A is easily accommodated if ComW acts to exclude σ^A from polymerase, to promote loading of σ^X onto core polymerase, or to provide extra free core enzyme.

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