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Adaptive Divergence in Experimental Populations of *Pseudomonas fluorescens*. III. Mutational Origins of Wrinkly Spreader Diversity

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ABSTRACT

Understanding the connections among genotype, phenotype, and fitness through evolutionary time is a central goal of evolutionary genetics. Wrinkly spreader (WS) genotypes evolve repeatedly in model Pseudomonas populations and show substantial morphological and fitness differences. Previous work identified genes contributing to the evolutionary success of WS, in particular the di-guanylate cyclase response regulator, WspR. Here we scrutinize the Wsp signal transduction pathway of which WspR is the primary output component. The pathway has the hallmarks of a chemosensory pathway and genetic analyses show that regulation and function of Wsp is analogous to the Che chemotaxis pathway from *Escherichia coli*. Of significance is the methyltransferase (WspC) and methylesterase (WspF) whose opposing activities form an integral feedback loop that controls the activity of the kinase (WspE). Deductions based on the regulatory model suggested that mutations within *wspF* were a likely cause of WS. Analyses of independent WS genotypes revealed numerous simple mutations in this single open reading frame. Remarkably, different mutations have different phenotypic and fitness effects. We suggest that the negative feedback loop inherent in Wsp regulation allows the pathway to be tuned by mutation in a rheostat-like manner.

UNDERSTANDING the relationship among DNA sequence, phenotype, and fitness through evolutionary time is a central goal of evolutionary genetics. Theoretical approaches, building on ideas first put forward by Fisher (1930) and Maynard Smith (1970), have led to a definition of the statistical properties expected to characterize the distribution of fitness effects among new mutations exposed to—and fixed by—selection (reviewed in Orr 2005b). However, these models say little about the specific kinds of mutational changes, in terms of the genetic or phenotypic basis of adaptive traits, expected in evolving populations. What is required is an understanding of the relationship (if any) between the underlying genetic architecture of an organism and the ability of the changes at the DNA sequence level to translate through to phenotypically useful solutions (Pepin *et al.* 2006).

Our work has focused on the ecological and genetic causes of diversity in simple laboratory populations of *Pseudomonas fluorescens* (strain SBW25). When propagated in spatially structured microcosms, populations of *P. fluorescens* rapidly diversify, giving rise to a range of niche specialist genotypes (Rainey and Travisano 1998). One common class of niche specialist colonizes the air–liquid interface of static broth microcosms and produces colonies with a characteristic wrinkled morphology on agar plates: these mutants are referred to as "wrinkly spreaders" (WS). WS morphs (of which there are many morphologically distinct types) arise by spontaneous mutation from the ancestral genotype and are substituted in the population because they enjoy a fitness advantage when rare. Their ability to colonize the air–liquid interface stems from the formation of a self-supporting mat that is a product of the combined (cooperative) activities of the individual cells (Rainey and Rainey 2003).

A single WS genotype, the *l*arge *s*preading *WS* (LSWS), has been the focus of genetic analysis. Suppressor studies have identified numerous genes that contribute either directly or indirectly to mat formation (Spiers *et al.* 2002; Gehrig 2005), but two loci are of special significance: *wss* and *w*rinkly *sp*reader (*wsp*)—transposon insertions throughout either locus abolish the wrinkled phenotype, niche specialization, and associated traits characteristic of LSWS (Spiers *et al.* 2002; Goymer *et al.* 2006).

The *wss* locus encodes proteins that together produce an acetylated cellulose polymer (Spiers *et al.* 2002, 2003). In LSWS, the *wss*-encoded enzymes are constitutively active as a consequence of overproduction of

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bis-(3′-5-)-cyclic dimeric guanosine monophosphate (c-di-GMP), an allosteric activator of cellulose (Ross *et al.* 1987; Goymer *et al.* 2006; Malone *et al.* 2007). Overproduction of c-di-GMP results in constitutive production of polymer, which, in conjunction with a similarly overproduced proteinaceous adhesin (Spiers *et al.* 2003), causes daughter cells to remain attached after cell division. The cellulosic polymer thus functions as a cell–cell glue and is the cause of cooperation among cells that form the WS mat (Spiers *et al.* 2002; Rainey and Rainey 2003).

The proximate cause of excess c-di-GMP in LSWS is *wsp*, a seven-gene operon that encodes a chemosensory pathway, although, more specifically, the cause is constitutive phosphorylation of the di-guanylate cyclase (DGC) response regulator WspR, the output component of the *wsp* pathway. When phosphorylated (at Asp67), the C-terminal DGC domain of WspR becomes functionally active and this leads to the joining of two molecules of GTP in a head-to-tail manner, resulting in the formation of c-di-GMP (Malone *et al.* 2007).

Here we define and genetically characterize the Wsp operon, predict mutational targets, and show that the cause of LSWS is a transversion in *wspF*. Analysis of *wspF* nucleotide sequence from another 26 independent WS genotypes showed that 13 harbor simple mutations. Remarkably, different mutations have different phenotypic and fitness effects. We suggest that these different effects are a consequence of the negative feedback loop inherent in Wsp regulation that allows the pathway to realize multiple output states.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and manipulation: The ancestral (wild-type) "smooth" (SM) strain is *P. fluorescens* SBW25 and was isolated from the leaf of a sugar beet plant grown at the University Farm, Wytham, Oxford, in 1989 (Rainey and Bailey 1996). The ancestral strain was placed at −80° immediately after isolation to minimize lab adaptation. The niche-specialist wrinkly spreader (LSWS) genotype (PR1200) was derived from the ancestral genotype following 3 days of selection in a spatially structured microcosm (Spiers *et al.* 2002). PR3522 (LSWSΔ*panB*) is a pantothenate requiring an auxotroph of LSWS (PR1200) and contains a clean deletion of the entire *panB* gene (Rainey 1999). The mutation is neutral in a pantothenate replete environment (Spiers *et al.* 2002). The WS genotypes WS_A–WS_Z are 26 independent WS genotypes obtained by propagating the ancestral SM genotype in 26 separate microcosms. After 5 days of selection, populations were diluted and plated onto KB agar, and a single WS genotype was selected at random from each microcosm. These genotypes were streaked onto a single colony to check purity and then stored at −80°.

P. fluorescens strains were cultured at 28° in Luria–Bertani (LB) (Miller 1972), Pseudomonas agar F (Difco), or King's medium B (KB) (King *et al.* 1954). *Escherichia coli* $DH5_a$, XL1-Blue MRF′ (Stratagene, La Jolla, CA), and S17-1λ_{pir} (Simon *et al.* 1983) were cultured at 37° in LB. Antibiotics were used at the following concentrations: ampicillin 100 μg ml^{-1}, kanamycin 75 μg ml^{-1} (50 μg ml^{-1} for *E. coli*), and tetracycline 25 μg ml^{-1}. Pantothenate was added to media at a final concentration of 0.24% (w/v) where required. CFC (Oxoid) was added to media to select for *P. fluorescens* strains following conjugation. Congo Red was used at a final concentration of 0.001% (w/v) in KB or LB agar without NaCl (NaCl causes Congo Red to precipitate). Calcofluor [Sigma (St. Louis) Fluorescent Brightener 28] was used in LB at 1–10 μM and cells or the polymer were incubated with the stain for 30 min before examination by fluorescent microscopy.

Plasmid DNA was introduced into *E. coli* and *P. fluorescens* by transformation (electroporation) or conjugation using standard procedures. The helper plasmid pRK2013 (Figurski and Helinski 1979) was used to facilitate transfer of plasmids between *E. coli* and *P. fluorescens*.

Molecular biology techniques: Plasmid DNA was isolated from *E. coli* using QIAprep plasmid kits (QIAGEN, Chatsworth, CA). Standard agarose gel electrophoresis and other recombinant DNA techniques were performed according to Sambrook *et al.* (1989). DNA fragments were recovered from agarose using a QIAEX II gel extraction kit (QIAGEN). pSK$^+$ (pBluescriptII SK$^+$, Stratagene), pCR2.1 (Invitrogen, San Diego), and pVSP61 (Loper and Lindow 1994) were used for cloning and overexpression of *wsp* genes. Allelic exchange and the construction of chromosomally integrated *′lacZ* operon fusions were performed using the suicide reporter plasmid pUIC3 (Rainey 1999). DNA sequencing was carried out on an ABI310 (Perkin-Elmer, Norwalk, CT) automated sequencer. Oligonucleotide primers used for sequencing, cloning, reverse transcriptase–PCR (RT–PCR), and construction of *wspA-F, wspC, wspF,* and *wspR* deletion strains are available upon request and are based upon the available SBW25 genome sequence (Sanger Centre).

Construction of deletion mutants, allelic replacements, and *lacZ* fusions: Deletion mutants were constructed as described previously (Rainey 1999). First, ~500 bp of DNA flanking either side of the gene (or genes) to be deleted were spliced together by PCR (Horton *et al.* 1989). The spliced DNA was cloned into pBluescript, from where it was sequenced to check for errors. The spliced fragment was then excised as a *Bgl*II–*Spe*I fragment and ligated into the suicide vector pUIC-3 (Rainey 1999). To generate *wspF* allelic replacements, PCR fragments of 3 kb centered on *wspF* were amplified from chromosomal DNA using *wsp*-specific primers and cloned into pCR2.1 (Invitrogen). *wspF* plasmids were reintroduced into *P. fluorescens* by electroporation and allowed to recombine with the chromosome by homologous recombination. Recombinants from which pCR2.1 had been lost were identified from LB agar plates after a brief period of nonselective growth in KB broth. The change in *wspF* sequence was confirmed by DNA sequencing. Chromosomal fusions between the last gene of the *wsp* operon (*wspR*) and *′lacZ* were generated by cloning the entire *wspR* gene as a *Bgl*II–*Spe*I fragment into pUIC3. The resulting plasmid was introduced into the genome by conjugation, and integration by a single homologous recombination event was ensured by selection for plasmid-encoded tetracycline resistance. Correct placement of the fusion was checked by PCR.

Fitness of genotypes: Competitive fitness of WS genotypes was determined by direct competition between each WS genotype and PR3522 (a *panB* deletion derivative of LSWS) and was performed in the presence of the ancestral SM genotype (the presence of the ancestral genotype ensured that the broth phase was colonized and this led to improved reproducibility of fitness measures). All strains were grown overnight in shaken KB broth (supplemented with pantothenate where appropriate) and competitors were introduced into spatially structured KB microcosms (~10^5 cells of each competitor) at a ratio of 1:1:1 (SM:LSWS Δ*panB*:WS_i). Relative fitness was

TABLE 1

Predicted function and domain characteristics of Wsp proteins

Protein	Size[a]	Predicted function	Pfam domain	*E*-value
WspA	547	MCP	HAMP	2×10^{-16}
			MCP	8.6×10^{-27}
WspB	170	Scaffold protein	CheW	7.2×10^{-27}
WspC	419	Methyltransferase	CheR	6.7×10^{-33}
WspD	232	Scaffold protein	CheW	4.5×10^{-26}
WspE	755	Histidine kinase/response regulator	Hpt	2.0×10^{-12}
			HATPase_c	9.1×10^{-22}
			CheW module	3.3×10^{-24}
			Response_reg	9.1×10^{-33}
WspF	336	Methylesterase	Response_reg	2.9×10^{-19}
			CheB_methylest	2.7×10^{-57}
WspR	333	Di-guanylate cyclase/response regulator	Response_reg	5.8×10^{-17}
			GGDEF	8.5×10^{-68}

[a] Number of amino acids.

calculated as the ratio of the Malthusian parameters of the two WS genotypes being compared (Lenski *et al.* 1991) and cell counts were determined after 24 hr of competition. The pantothenate-marked gentoype was distinguished from WS_i by plating cells on vitamin-free KB agar supplemented with 4.8×10^{-6}% pantothenic acid (on this medium the pantothenate-marked strain is readily distinguished by its greatly reduced size). All cultures were whirli-mixed for 60 sec before dilution plating to maximally disperse clumped cells.

RESULTS

Defining the Wsp chemosensory pathway: The *wsp* locus spans ~8.5 kb and is composed of seven open reading frames designated *wspABCDEFR*. Analysis of whole-genome sequence data shows that *wsp* is a highly conserved feature of all Pseudomonas genomes (Stover *et al.* 2000; Nelson *et al.* 2002; Feil *et al.* 2005; Joardar *et al.* 2005; Paulsen *et al.* 2005): it is also resident on plasmid pMLb from *Mezorhizobium loti* (Kaneko *et al.* 2000). While effects of disrupting Wsp function have been observed in *P. aeruginosa* (D'Argenio *et al.* 2002; Hickman *et al.* 2005) and *P. fluorescens* (Spiers *et al.* 2002), the locus has received little direct characterization: in lieu of a genetic model of Wsp regulation, we briefly describe the salient features of each predicted protein (Table 1).

Predicted proteins of the Wsp locus: WspA (AAO92333) resembles a membrane-associated methyl-accepting chemotaxis protein (MCP). It contains the functional domains of a typical chemotactic transducer (Falke *et al.* 1997), including an N-terminal (periplasmic) sensory ligand-binding region flanked by two membrane-spanning regions; a C-terminal cytoplasmic domain including the conserved signaling region and methylation sites; a C-terminal domain composed of two subdomains—the conserved HAMP domain involved in regulation of receptor methylation (Chervitz and Falke 1996); and the conserved methyl-accepting domain containing MCP methylation sites (Le Moual and Koshland 1996).

WspB (AAO92334) and WspD (AA092336) are predicted scaffold proteins similar to CheW from *E. coli* (Levit *et al.* 2002). WspD has, in addition to a CheW domain, another 60 amino acids at the N terminus.

WspC (AA092335) contains a conserved N-terminal CheR domain and is a probable methyltransferase (Djordjevic and Stock 1997). Unlike CheR, WspC contains an additional 160 residues downstream of the CheR domain, including amino acid sequences that define a tetratrico peptide repeat region. The methyltransferase homolog FrzF of *Myxococcus xanthus* (McCleary *et al.* 1990) shows a similar arrangement.

WspE (AA092337) contains regions homologous to both the CheA histidine kinase domain and the CheY response regulator domain. Overall, WspE shows the greatest structural homology to the FrzE hybrid protein of *M. xanthus* (McCleary and Zusman 1990). The CheA-like domain of WspE comprises three distinct modules: the Hpt module, which contains key residues involved in autophosphorylation of the kinase; the conserved catalytic module responsible for kinase activity; and a module essential for receptor-mediated regulation (which contains the CheW and receptor-binding sites).

WspF (AA092338) has two predicted domains: an N-terminal CheY response regulator domain (Stock *et al.* 2000) and a CheB methylesterase domain. Overall, it is similar to CheB from *E. coli* (see supplemental Figure 3 at http://www.genetics.org/supplemental/), which is an esterase that hydrolyzes the methyl esters formed by CheR and restores negatively charged glutamate residues on MCPs (West *et al.* 1995). The N-terminal response regulator receiver domain contains the major active-site aspartate residues (Asp-10, Asp-11, and Asp-56—the

phosphorylation site) (Stock and Surette 1996; Stock *et al.* 2000). The C-terminal methylesterase domain contains the major methylesterase active sites (Ser-164, His-190, and Asp-286) (West *et al.* 1995).

WspR (AAL71852) is a DGC response regulator and has been described in detail elsewhere (see Goymer *et al.* 2006). Like WspE and WspF, it also contains (at its N terminus) a CheY response regulator domain; the C-terminal domain is a DGC whose function is dependent upon phosphorylation of Asp67 (Goymer *et al.* 2006).

The seven *wsp* genes form a single transcriptional unit: The first six genes of the *wsp* locus are translationally coupled (the stop codon of the preceding gene overlaps the predicted start codon of the following gene), indicating that *wspA-F* form a single transcriptional unit; however, a 50-nucleotide gap separates the seventh gene *wspR* from *wspF*, suggesting the possibility that *wspR* is a separate transcriptional unit. An RT–PCR strategy was used to test this hypothesis; specifically, mRNA was extracted from the ancestral genotype and cDNA was generated by RT–PCR using primers flanking the 3′-end of *wspF* and the 5′-start of *wspR* (see supplemental Figure 1 at http://www.genetics.org/supplemental/ for details). A series of test and control PCR reactions were then performed to determine whether cDNA corresponding to the sequence between *wspF* and *wspR* had been generated (as expected if *wspF* and *wspR* were transcribed as a single mRNA). A 0.5-kb fragment was obtained using primers that flank the *wspF–wspR* intergenic region, demonstrating that *wspR* is part of the same transcriptional unit as *wspF*. DNA sequence of this fragment confirmed its identity.

Transcription of *wsp* is similar in SM and LSWS: It was not inconceivable that the cause of enhanced WspR activity in LSWS (*vs.* SM) was a mutation in the *wsp* promoter that caused enhanced transcription of the operon. A promoterless *lacZ* fusion was therefore made to *wspR* (without affecting the *wspR* open reading frame) and integrated into the genome of both the ancestral SM and derived LSWS genotypes. β-Galactosidase activity was determined in King's medium B after 4, 12, 24, and 48 hr of growth. The *wsp* operon was transcriptionally active at all stages of growth and, while expression was slightly lower in the ancestral genotype, typically the difference was less than twofold (supplemental Figure 2 at http://www.genetics.org/supplemental/). Such minor differences were not considered significant and the possibility that the cause of LSWS was a mutational change in the promoter region was not further investigated.

The Wsp pathway donates phosphoryl groups to WspR: Previous analysis of WspR variants [generated by *in vitro* means (Goymer *et al.* 2006)] showed (1) that phosphorylation of WspR was necessary for development of the WS phenotype, (2) that in LSWS WspR was constitutively phosphorylated, and (3) that the source of phosphoryl groups in LSWS was "upstream" of WspR. The latter finding stemmed from the analysis of WspR variants with dominant-negative effects, that is, variants that, when overexpressed in LSWS, caused development of the ancestral SM phenotype. Sequence (and genetic) analysis showed that these variants harbored single nonsynonymous amino acid substitutions in the C-terminal DGC domain. The dominant-negative effect was thus attributed to competitive inhibition: variant forms with inactive DGC domains (being in excess) "soaked up" phosphoryl groups that were otherwise destined to activate the DGC activity of the chromosomally encoded WspR. This result indicated that one effect of the mutation(s) responsible for LSWS was overstimulation of a kinase (Goymer *et al.* 2006). The most likely candidate is WspE, but cross talk among two-component sensing systems meant that kinases beyond the Wsp operon might also be involved.

To test the hypothesis that the Wsp pathway is the source of phosphoryl groups, the first six genes of the *wsp* operon were deleted from the LSWS genotype. The deletion was constructed in such a way as to ensure minimal disruption to *wspR*; indeed, the ATG start codon of the first gene of the *wsp* operon (*wspA*) became the start codon of *wspR* so that *wspR* remained fully under the control of the *wsp* promoter. The resulting mutant expressed the ancestral smooth morphology, was unable to colonize the air-liquid interface of static broth microcosms, and no longer produced detectable amounts of cellulose as determined by both Congo Red assay and fluorescent microscopy of calcofluor-stained cells. This result confirms Wsp as the source of phosphoryl groups.

Regulation of Wsp: The discovery that Wsp is the source of phosphoryl groups led to questions regarding Wsp function and regulation. As demonstrated above (Table 1), the Wsp operon bears substantial similarity to the chemotaxis (Che) operon from *E. coli*: as such, Che provides the basis of a working model.

Che is a complex two-component signal transduction system found in almost all motile bacteria: it has been extensively investigated and reviewed (Stock and Surette 1996; Falke *et al.* 1997; Stock *et al.* 2000; Baker *et al.* 2006). Its primary function is to sense and process environmental stimuli in such a way as to effect change in the direction of rotation of the flagella motor. Central to the pathway are two proteins: CheA (histidine kinase) and CheY (response regulator). CheA catalyzes the transfer of phosphoryl groups from ATP to a conserved histidine residue on the kinase, while CheY catalyzes the transfer of the phosphoryl group from the kinase to a conserved aspartate residue on the response regulator. When phosphorylated, CheY associates with the flagella motor, causing a reversal in the direction of rotation of the flagella bundle. Activity of CheY is also affected by CheZ, a CheY-specific phosphatase (there is no homolog of CheZ in the Wsp pathway). CheA does not act alone, but forms a membrane-associated receptor complex in

conjunction with MCPs and the scaffold protein CheW; the receptor complex integrates sensory information to control the level of CheA kinase activity.

Two additional proteins play a critical role in regulating kinase activity: CheR and CheB. Even in an environment devoid of stimulatory ligands, the kinase switches between active and inactive states about once every second due to the opposing activities of CheR and CheB. CheR constantly adds methyl groups to glutamate residues on the cytoplasmic domain of the MCP to the point that the kinase becomes activated; phosphoryl groups pass to CheY (which dissociates from the signaling complex to interact with the flagella motor) and also to CheB. Upon activation, CheB removes methyl groups and thus resets the kinase to an inactive state.

Given that the Wsp operon has proteins with apparent functional equivalence to Che, a model for Wsp function and regulation was developed (Figure 1). Initial attempts to test the model by genetic complementation of a set of defined *E. coli che* mutants (*cheA*, *cheW*, *cheR*, *cheB*, and *cheY*) using predicted homologs from the Wsp pathway (*wspE*, *wspB/D*, *wspC*, *wspF*, and *wspR*) met with minimal success (Bantinaki 2002).

An alternative approach was to test directly the regulatory model by genetic manipulation of the component parts. If the model is correct (and the individual components are functionally equivalent to their Che counterparts), then activity of the output component, WspR, should be dependent upon the activity of the kinase (WspE), which should ultimately be dependent upon the relative activities of the methyltransferase (WspC) and the methylesterase (WspF). It follows, then, that an increase in the level of expression of WspC relative to WspF (but not the reverse) in the ancestral SM genotype should generate the WS colony morphology because an increase in WspC activity relative to WspF will cause methylation of glutamate residues on the MCP to rise to an irreversibly high level, resulting in constitutive activation of the kinase, overactivation of WspR, production of excess c-di-GMP, overactivation of the cellulose biosynthetic enzymes, and a switch in colony morphology from SM to WS (with concomitant effects on cellulose biosynthesis and niche specialization). Conversely, an increase in the level of expression of WspF (relative to WspC) in the derived LSWS genotype should cause development of the ancestral smooth morphology because an increase in WspF activity relative to WspC (but not the reverse) will cause modified glutamate residues on the MCP to be hydrolyzed, leading to a negligible number of modified glutamate residues and thus inactivation of the WspE kinase, inactivation of WspR, no synthesis of c-di-GMP, inactivation of cellulose biosynthetic enzymes, and a switch in colony morphology from LSWS to SM (with concomitant effects on cellulose biosynthesis and niche specialization).

To test these predictions, *wspC* and *wspF* were deleted from both ancestral SM and derived LSWS backgrounds. In addition, both genes were overexpressed (separately) from the constitutive *lac* promoter of plasmid pVSP61—in both ancestral SM and LSWS backgrounds. The results, shown in Table 2, are fully consistent with predictions of the model: deletion of *wspF* and overexpression of *wspC* in the ancestral SM background caused development of the wrinkled colony morphology (no effect on colony morphology occurred when *wspC* was deleted or *wspF* was overexpressed). Conversely, the wrinkled morphology of

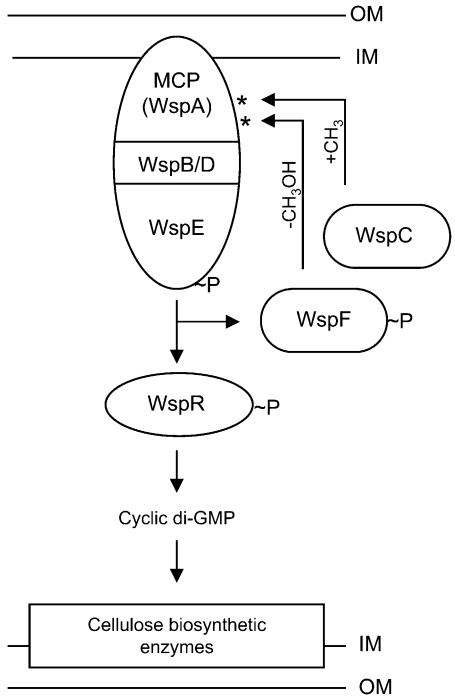

Figure 1.—Model for the regulation and function of the Wsp pathway based on the Che (chemotaxis) pathway of enteric bacteria. The MCP (WspA), scaffold proteins (WspB and WspD), and kinase (WspE) form a membrane-bound receptor-signaling complex (IM, inner membrane; OM, outer membrane). WspR is a DGC response regulator and the primary output of the pathway. When phosphorylated, WspR catalyzes the synthesis of cyclic-di-GMP, an allosteric activator of cellulose biosynthetic enzymes. The level of kinase activity is controlled by the opposing activities of methyltransferase (WspC) and methylesterase (WspF), which add and remove (respectively) methyl groups from conserved glutamate residues on the signaling domain of the MCP (asterisks). WspC is constitutive, whereas WspF becomes active upon receipt of phosphoryl groups from the kinase. Addition of methyl groups to glutamate residues on the MCP by WspC causes the kinase to autophosphorylate and results in activation of WspF (and WspR); phosphorylated WspF removes methyl groups and thus resets the WspE kinase to an inactive state. As in the Che pathway of *E. coli*, the model presented here predicts that Wsp continually oscillates between active and inactive states. According to the model, a mutation in WspF that decreases WspF function (or removes functionality altogether) will result in constitutive activation of the kinase, constitutive activation of WspR, overproduction of c-di-GMP, and overproduction of cellulose and other adhesive polymers, resulting in expression of the WS phenotype.

TABLE 2

Effect of manipulation of components of the Wsp pathway on colony morphology of ancestral SM and derived LSWS genotypes

Genotype and colony morphology of initial genotype	Genetic manipulation[a]	Effect on colony morphology
SM	Δ*wspC*	SM
SM	Δ*wspF*	WS[b]
SM	Δ*wspR*	SM
SM	pVSP61-wspC	WS[b]
SM	pVSP61-wspF	SM
SM	pVSP61-wspR	WS[b]
SM	pVSP61-wspE	SM
LSWS	Δ*wspC*	SM[b]
LSWS	Δ*wspF*	WS
LSWS	Δ*wspR*	SM[b]
LSWS	pVSP61-wspC	WS
LSWS	pVSP61-wspF	SM[b]
LSWS	pVSP61-wspR	WS
LSWS	pVSP61-wspE	SM[b]

[a] Manipulation involved either deletion of a gene (e.g., Δ*wspC*) or overexpression of the gene on plasmid pVSP61 (*e.g.*, pVSP61-*wspC*).
[b] Conversion of phenotype.

LSWS converted to smooth when *wspF* was overexpressed and the same conversion was evident upon deletion of *wspC* (no phenotypic changes were observed when *wspF* was deleted or when *wspC* was overexpressed). By way of positive controls, the output component WspR was deleted from SM and derived LSWS and overexpressed in the same two genotypes, and results were consistent with previous findings, namely, that deletion of the *wspR* in LSWS causes development of SM and that overexpression of *wspR* in SM causes development of the wrinkled morphology typical of WS (Goymer *et al.* 2006). The kinase WspE was also cloned and overexpressed on the basis that overexpression might result in an increase in spontaneous autophosphorylation and thus cause SM to convert to WS; however, this did not occur. Interestingly, however, overexpression of *wspE* in LSWS caused reversion to the SM phenotype. This effect is likely to be attributable to a disruption of the receptor-signaling complex due to an imbalance between the various components.

Predicting the mutational cause of LSWS: The model established for signal transduction through the Wsp pathway allowed for predicting the likely mutational causes of the LSWS genotype. From the very beginnings of the genetic analysis of WS it had been difficult to reconcile the genetic data on the origins of the WS phenotype with the repeatable occurrence of WS mutants in static broth microcosms. The genetic data are consistent with the WS phenotype resulting from a "gain of function" (Spiers *et al.* 2002), yet the relative ease by which WS mutants arise (estimated at $\sim 5 \times 10^{-7}$) suggests that the underlying mutation is a loss of function. The model outlined in Figure 1 (and tested above) indicates that WS genotypes could indeed arise by a loss-of-function mutation: a mutation that decreased or abolished the function of the methylesterase WspF would result in overstimulation of the WspE kinase (due to ever-increasing levels of glutamate modification caused by WspC), with ensuing positive effects on WspR activity, c-di-GMP production, and production of adhesive polymers. Conversely, while mutations causing WS could arise within the methyltransferase WspC—or within WspR itself—that increase activity of either component, these would have to be gain-of-function (or at least increase-in-activity) mutations and thus should be rare. To test these predictions, the nucleotide sequence was obtained from *wspF*, *wspC*, and *wspR* from both SM and the derived LSWS genotype. No change was found in *wspC* or *wspR*; however, a single nucleotide change (A901C) (causing a nonsynonymous amino acid change: S301R) was found in *wspF* of LSWS.

Many independent mutations occur within *wspF*: Independent populations evolving in identical environments often show parallel genetic changes and the fact that they do is a strong argument for the changes being adaptive (Cooper *et al.* 2001, 2003; Zhong *et al.* 2004; Colosimo *et al.* 2005; Woods *et al.* 2006). A collection of 26 independently obtained WS was thus analyzed for changes to the nucleotide sequence of *wspF*. The results presented in Table 3 show that 13 of the 26 WS genotypes contained simple mutations (transitions, transversions, or short deletions) in *wspF*; WS_N harbored the same genetic change as was found in LSWS (*wspF* A901C), but all other mutations were unique. A careful analysis of the nucleotide sequence of *wspF* failed to reveal any evidence of mutable nucleotide tracts, such as repeats or insertion elements. For those WS genotypes that did not contain a mutant *wspF* allele, the nucleotide sequence was obtained from *wspC* and *wspR*, but no changes in these genes were detected.

Substantial structure–function analyses of the homologous methylesterase CheB (West *et al.* 1995; Stock and Surette 1996; Djordjevic *et al.* 1998) have defined domain structure, active sites, and catalytic residues (see above and supplemental Figures 3 and 4 at http://www.genetics.org/supplemental/). Analysis of the particular mutations in light of this knowledge (Table 3) indicates a range of effects from seemingly catastrophic in the case of WS_O [WspF V79Δ(6)], which lacks the C-terminal two-thirds of the protein, to seemingly minor in the case of WS_E (WspF V220L), which differs from wild type by a single amino acid with similar chemical properties.

Allelic replacements confirm the adaptive significance of *wspF* mutations: While parallel genetic changes in *wspF*, combined with a clear understanding of the physiological effects of defects in methylesterase function, provide a persuasive case for the mutations in *wspF* being

TABLE 3

***wspF* mutations from independent WS genotypes**

WS genotype[a]	Nucleotide change	Nature of mutation	Amino acid change	Predicted effect on function and group[b]
LSWS	A901C	Transition	S301R	Reduced activity (2); mutation close to active site
WS_A	T14G	Transversion	I5S	Reduced activity (2); mutation close to active site
WS_B	Δ620-674	Deletion	P206Δ(8)[c]	No activity (3); C-terminal third of protein lost
WS_C	G823T	Transversion	G275C	Reduced activity (2); mutation close to active site
WS_E	G658T	Transversion	V220L	Reduced activity (2)
WS_F	C821T	Transversion	T274I	Reduced activity (2); mutation close to active site
WS_G	C556T	Transversion	H186Y	No activity (3); catalytic residue altered
WS_J	Δ865-868	Deletion	R288Δ(3)[c]	Reduced activity (2); most of protein remains
WS_L	G482A	Transition	G161D	Reduced activity (2)
WS_N	A901C	Transition	S301R	Reduced activity (2); same mutation as in LSWS
WS_O	Δ235-249	Deletion	V79Δ(6)[c]	No activity (3); C-terminal two-thirds lost
WS_U	Δ823-824	Deletion	T274Δ(13)[c]	No activity (3); Asp286 active site residue is lost
WS_W	Δ149	Deletion	L49Δ(1)[c]	No activity (3); most of protein lost
WS_Y	Δ166-180	Deletion (in frame)	Δ(L51-I55)	Reduced activity (2); small in-frame deletion of five residues

[a] No *wspF* (*wspC* or *wspR*) mutation was detected in WS_D, WS_H, WS_I, WS_K, WS_M, WS_P, WS_Q, WS_R, WS_S, WS_T, WS_V, WS_X, and WS_Z.
[b] Defects caused by each mutation were scored on the basis of the likely magnitude of the effect (see text): 1, wild-type *wspF* (13 isolates); 2, reduced activity (9 isolates); 3, no activity (5 isolates).
[c] P206Δ(8) indicates a frameshift; the number of new residues before a stop codon is reached is in parentheses.

causal, we sought direct evidence by recreating three different *wspF* mutations (LSWS *wspF* A901C, WS_A *wspF* T14G, and WS_G *wspF* C556G) in the ancestral genotype.

Replacement of the wild-type allele in the ancestral SM genotype by each variant *wspF* allele in independent allelic replacement experiments resulted in genotypes with WS phenotypes. In each instance, the recreated WS genotype bore the subtle, but nonetheless distinct, phenotypic characteristics unique to the WS genotype from which the mutant allele was obtained. In addition, detailed proteomic analysis reported elsewhere shows that 52 proteins differentially expressed between the ancestral and derived LSWS genotypes showed consistent changes in the recreated *wspF* A901C genotype (Knight *et al.* 2006). To further examine the evolutionary significance of the *wspF* A901C mutation (in LSWS), this mutant allele was replaced with the wild-type sequence (*wspF* C901A): in all phenotypic respects this genotype manifested the ancestral SM type.

While analysis of the phenotypic effects of different *wspF* alleles has made clear the connection between the nucleotide sequence of *wspF* and phenotype, the relationship between the *wspF* allele and fitness effects required investigation. To this end, the fitness of each recreated WS mutant was compared (in triplicate assay) to the fitness of the original WS genotype (LSWS, WS_A, WS_G) from which the *wspF* allele was originally obtained (in each case fitness was determined relative to LSWS *panB* and in the presence of the SM ancestor; see materials and methods). In each instance the fitness of the original WS genotype and the recreated WS genotype was not significantly different [$F_{1,17} = 0.100$, $P = 0.760$]. Taken together, these data provide strong evidence that the mutations identified in *wspF* are solely responsible for both the phenotypic and fitness effects of these WS genotypes.

Different mutations in *wspF* have a range of fitness effects: The class of adaptive mutant referred to as WS encompasses an extensive range of diversity at both phenotypic and fitness levels. Just how a single genome can give rise to such a broad repertoire of diversity is an enigma. A clue was provided in a previous study (Goymer *et al.* 2006): variation in both form and fitness of WS morphs could be generated by alterations in the level of activity of the DGC regulator WspR.

With knowledge of the mutational origins of 14 independent WS—and being in possession of a further 13 WS genotypes with no mutational change in *wspF*—we sought understanding of the extent to which DNA sequence variation in *wspF* corresponds to variation in competitive fitness by determining the fitness of the independently derived wrinkly spreader genotypes in competition against the LSWS containing a neutral genetic marker (Δ*panB*) in the presence of the SM ancestor.

Substantial quantitative genetic variation in fitness was observed among the entire set of independently derived wrinkly spreader genotypes (Figure 2) and this was shown to be highly significant by ANOVA [$F_{26,79} = 52.81$, $P < 0.0001$]. A significant difference in fitness was also evident among only those WS genotypes with mutations in *wspF* (*i.e.*, genotypes with no *wspF* mutation were removed from the analysis) [$F_{13,42} = 60.65$, $P < 0.0001$]. Further analysis revealed that neither knowledge of the different kinds of mutations within *wspF* (deletion, transition, or transversion) nor the predicted activity of the *wspF* protein (Table 3) were capable of

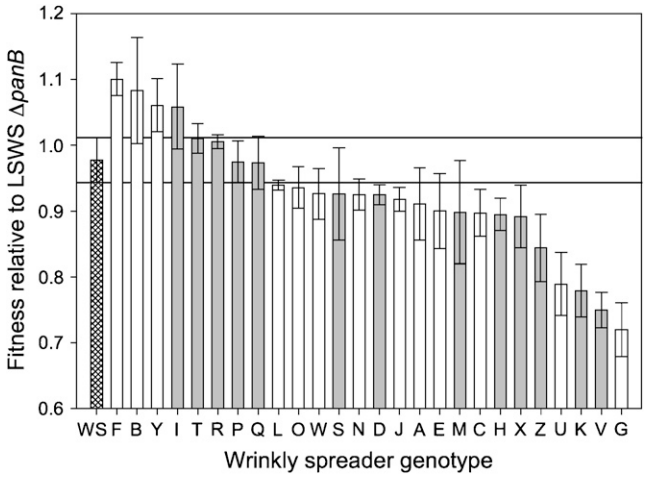

FIGURE 2.—Fitness of the 26 independently isolated wrinkly spreader mutants relative to Δ*panB*-marked LSWS (hatched bar). Fitness was measured in static broth microcosms over the course of 24 hr. A fitness of 1.0 means that the fitness is identical to LSWS Δ*panB*. Open bars represent strains with mutations within *wspF*; shaded bars represent mutants that are wild type for *wspF*. Error bars are means and 95% confidence intervals from four replicate assays. In a separate experiment, the fitness of a defined *wspF* null mutant (SM Δ*wspF*) was determined relative to LSWS Δ*panB*: the fitness of SM Δ*wspF* was indistinguishable to WS_G (see text).

explaining significant amounts of genetic variation in fitness [type of mutation (deletion, transition, transversion): $F_{2,11} = 0.14$, $P = 0.87$; predicted effect on protein activity (reduced or inactive; Table 3): $F_{1,12} = 1.44$, $P = 0.25$ (nested ANOVA with genotypes, nested within the main effect of mutation type or predicted function, used as the error term)]. These data show that subtle, and usually small, changes in DNA sequence within the coding region of a single gene produce quantitative variation at the level of fitness that cannot be predicted on the basis of the particular kind of mutation nor the predicted activity of the resulting protein.

Given the lack of correspondence between the *wspF* mutation and ensuing fitness effects, it was of interest to determine the fitness consequences of a clearly defined *wspF* null mutation. To this end, the fitness of three independently generated SM Δ*wspF* genotypes (which are phenotypically WS; see above) was determined relative to the *panB*-marked LSWS genotype (in triplicate assay). In the same experiment, the fitness of independent WS genotypes WS_G and WS_B was also measured (as representative of WS genotypes with low and high fitness, respectively). Genotypes WS_G and WS_B showed qualitatively similar fitness to those reported in Figure 2 and ANOVA revealed a significant difference among means [$F_{4,10} = 11.87$, $P = 0.0008$]. Tukey's honestly significantly different (HSD) test showed that the fitness of WS_G was significantly less than the fitness of WS_B ($P < 0.05$), but indistinguishable from the three SM Δ*wspF* genotypes ($\alpha = 0.05$). This indicates that a true *wspF* null mutation produces a WS genotype with a fitness (relative to LSWS) that is at the low end of the spectrum of WS fitness measurements.

DISCUSSION

Two decades ago one of us became fascinated with the adaptive radiation that occurs each time Pseudomonas populations are propagated in spatially structured microcosms. Examination of an agar plate displaying the net outcome of such a radiation reveals an abundance of ecologically significant diversity, elegant in form and with different types seemingly fitting into a multiplicity of niches (see RAINEY and TRAVISANO 1998). Noting both the pace of diversification (a mere 50 generations) and the degree of fit between different types and their environment, it is tempting to suggest that the radiation is genetically programmed. While analysis is yet to extend beyond the WS class of variant, the evidence presented here indicates that the diverse array of WS types arises as a consequence of nothing more than simple, random (spontaneous) mutation aided by intense diversifying selection.

While the mutations are simple, their effects are both phenotypically and ecologically profound. A functionally compromised WspF methylesterase results in constitutive activation of the WspR DGC (GOYMER *et al.* 2006), overproduction of c-di-GMP (MALONE *et al.* 2007), and enhanced production of adhesive substances (SPIERS *et al.* 2002, 2003; SPIERS and RAINEY 2005), most importantly, of a partially acetylated cellulose polymer (SPIERS *et al.* 2002). The net effect is to cause daughter cells to remain attached after cell division. The cellulosic polymer thus functions as a cell–cell glue and is the cause of cooperation among cells that form the WS mat (RAINEY and RAINEY 2003). Given appropriate ecological conditions, WS genotypes follow distinctively new evolutionary trajectories compared to the ancestral type. The potential for the evolution of further complexity at the group level is of considerable interest and possibility.

Predicting adaptive evolution: Predicting the course of evolutionary change is the holy grail of evolutionists (ORR 2005a). On the basis of a variety of different experimental and theoretical approaches, accurate predictions have been made in a small number of instances. For example, a phylogenetic analysis of nucleotide polymorphisms within the influenza hemagglutinin gene enabled accurate predictions of the mutant lineage most likely to persist in the following year (BUSH *et al.* 1999). Similarly, using a biochemical model of lactose flux developed for *E. coli* populations inhabiting lactose-limited chemostats, DEAN (1989) and colleagues (DEAN *et al.* 1986; DYKHUIZEN *et al.* 1987) predicted the target of selection—the lactose permease (ZHANG and FERENCI 1999). More recently, mutational trajectories leading to enhanced levels of antibiotic detoxifying enzymes have

enabled detailed predictions as to the future evolution of antibiotic resistance (Barlow and Hall 2003; Hall and Barlow 2004; Weinreich *et al.* 2006).

Knowing that complete elimination of the methylesterase activity of WspF was sufficient to generate the WS phenotype, combined with the fact that most mutations are deleterious (Kimura 1968), led to the prediction that the evolutionary cause of LSWS was a mutation in *wspF*. This proved to be correct. While gain-of-function mutations in other components of the Wsp pathway might reasonably cause WS—*e.g.*, a mutation in WspC that leads to enhanced methyltransferase activity or a mutation in WspR that relieves N-terminal inhibition of the DGC domain (Goymer *et al.* 2006)—such mutations are expected to be rare, relative to the *wspF* loss-of-function mutations. Indeed, in this study, of those additional components of the Wsp pathway that were sequenced (in those WS genotypes that harbored no mutation in *wspF*), no mutations were detected.

The genetics of phenotypic innovation: While there is interest in predicting the future course of evolution, attention has also been devoted to understanding the nature of mutations responsible for major fitness effects, for example, those responsible for developmental innovations among multicellular organisms (Raff 1996; Carroll 2005). There is general acceptance of the conjecture that major phenotypic innovations are likely to arise from changes in gene regulation and particularly from changes in transcriptional control (King and Wilson 1975). Indeed, the literature of evolutionary developmental biology is replete with examples that show that changes in transcriptional regulation, particularly of *cis* regulatory sequences (Stern 2000), are a significant source of evolutionary novelty (Doebley *et al.* 1997; de Rosa *et al.* 1999; Simpson 2002; Levine and Tjian 2003; Wray 2003; Shapiro *et al.* 2004).

The genetic changes responsible for the evolution of WS share two significant similarities with these "major mutations." First, the phenotypic effects have important ecological implications and are macro-evolutionary in terms of effects (although wrought by the simplest of possible mutations). Second, *wspF* mutations change the level of activity of an existing pathway and are thus regulatory in nature. However, there is an important difference: the mutations in *wspF* exert their primary effect at a post-translational level. That their effect manifests at this level shows that changes at "alternate regulatory levels" can be evolutionarily significant (Alonso and Wilkins 2005).

Arguably, there is a third similarity: the net effect of the *wspF* mutations is the generation of a phenotype that is not achievable by the ancestral genotype. The mutation does more than simply result in constitutive expression of a trait that the ancestral type manifests given an appropriately intense stimulatory signal. The firmness of this statement is based on an understanding of Wsp regulation (and particularly of the integral control system composed of WspF and WspC) and is discussed more fully below, but this work adds to the growing body of evidence that shows that the range of phenotypic possibilities arising from relatively simple alterations in pathway control can be difficult to predict and highly innovative.

Genetic architecture of Wsp: Central to the problem of explaining variation is an understanding of the relationship between the underlying genetic architecture of an organism and the ability of changes at the DNA sequence level to translate through to phenotypically useful solutions. Among the more significant challenges posed by the *P. fluorescens* radiation is an explanation for the array of phenotypic diversity evident within the WS class of mutants. The discovery of multiple independent mutations within *wspF* with a range of different fitness effects suggests that the pathway (and the structural components that it regulates) can accommodate the production of heritable phenotypic variation. Received wisdom says that nonsynonymous mutations in a single gene ought to have a limited spectrum of effects: most are expected to abolish function and thus different mutations should generate phenotypically equivalent outcomes. Two interrelated factors may be significant in terms of an explanation for the system's capacity to generate phenotypic novelty: first, the connectivity of the Wsp signaling pathway and, second, the negative regulatory function of WspF.

The Che pathway of *E. coli* is the most thoroughly studied of any signal transduction cascade: not only is the function of the individual components well understood, but also analysis of the system as a whole has revealed important principles of network design. In particular, the Che pathway represents "a minimal topology providing high robustness to physiological perturbation" (Kollmann *et al.* 2005, p. 507)—a design principle that appears to be widespread among characterized Che pathways from a range of organisms (Szurmant and Ordal 2004), including pathways in eukaryotes under identical control (Raser and O'Shea 2004). Central to this robustness is integral feedback control, control that is defined by the combined activities of methyltransferase (CheR/WspC) and methylesterase (CheB/WspF). Together, these two components ensure that the difference between the actual output and the steady-state conditions are fed back into the system (Yi *et al.* 2000).

While Wsp does not function to control chemotaxis, the functional equivalence among Che and Wsp components, and particularly the experimentally demonstrated integral feedback loop, indicates that Wsp conforms to the same robust design principles. Attention has been drawn to a fundamental tension between a robust design and evolvability (the capacity to generate heritable phenotypic variation); the seemingly problematic nature of this tension has led to the development of complex arguments and theoretically tortuous

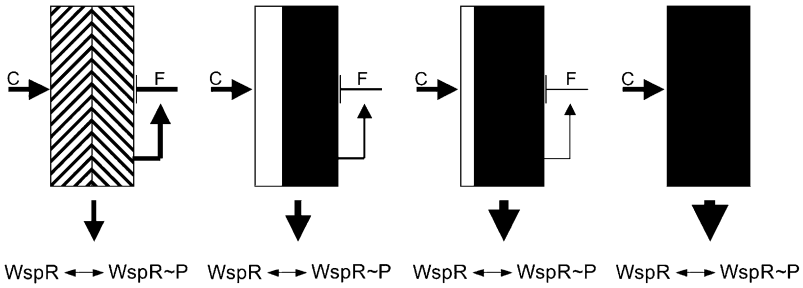

FIGURE 3.—A model to account for the effect of *wspF* mutations on the signaling status of the Wsp pathway. The Wsp signaling machinery (WspA, WspB, WspD, WspE) is shown as a box with the letters C and F indicating the proteins WspC (methyltransferase) and WspF (methylesterase), respectively. The links indicate activations (arrows) or repressions (bar ends). In the absence of a stimulatory ligand, the signaling machinery oscillates between an active (solid) and inactive (open) state, depending on the combined (and opposing) effects of WspC and WspF, which cause the cell to achieve a particular balance between phosphorylated and nonphosphorylated WspR. (Left) The signaling state of the wild-type Wsp pathway that spends time in both active (upward diagonal lines) and inactive (downward diagonal lines) states. Mutations in *wspF* that reduce protein function, even just slightly, destroy the capacity of the pathway to fluctuate between activity states, producing instead a steady-state output (a constant level of phosphorylated WspR). The precise strength of the output signal depends on the degree to which WspF function is reduced and is maximal when WspF function is completely eliminated (right). Different mutations are predicted to tune the pathway to different output levels (middle panels).

solutions (*e.g.*, WAGNER 2005a,b). We see no *a priori* reason for such a tension.

In the case of Wsp, the integral feedback loop is critically important to the pathway's capacity to accommodate the generation of phenotypic novelty. Drawing upon knowledge of Che function, the activity state of Wsp (even in the absence of a stimulatory ligand) is expected to fluctuate rapidly between active and inactive states. Even when maximally stimulated, the pathway will never deliver a constant output signal because the opposing activities of WspF (CheB) and WspC (CheR) constantly reset the signaling status of the kinase. Given the critical control exerted by WspF-WspC, it is not unreasonable to expect function of this feedback loop to be especially sensitive to genetic changes that affect the capacity of either component to balance the activity of the other. Moreover, any such disturbance, even a very minor one, will destroy the capacity of the pathway to fluctuate between activity states, producing instead a steady-state output. The precise strength of the output signal will depend on the degree to which WspF function is reduced and will be maximal when WspF function is completely eliminated. Put another way, the connectivity of the pathway is such that the output signal from the pathway can be converted from an oscillatory to a steady-state signal and the precise level can be tuned by mutation in a rheostat-like manner (Figure 3). While this model requires experimental validation, it would appear that Wsp is robust to physiological change, and yet fragile to genetic change, while at the same time possessing a topology and connectivity to other cellular components that leads to this genetic fragility manifesting as heritable phenotypic variation.

Phenotype–genotype map: Our model predicts a correlation between the fitness effects of *wspF* mutations and the extent to which the mutations compromise the function of the enzyme. The fact that the fitness of the *wspF* deletion strain is at the lower end of the spectrum of fitness effects indicates that the relationship between mutational effect and fitness is an inverse one. While this relationship holds for some WS genotypes, it by no means holds for all. For example, the fitness of WS_G and WS_U [WspF H186Y and WspF T274Δ(13), respectively] was among the lowest of all WS genotypes and is consistent with the fact that these mutations abolished predicted active sites and therefore should abolish function. However, WS_O [V79Δ(6)] carries a deletion that causes a frameshift such that two-thirds of the protein is expected to be absent and yet this genotype has an intermediate fitness. A number of possible explanations can be offered. First, having performed allelic exchange analyses of only three WS genotypes, it is not possible to rule out the possibility that some WS genotypes harbor additional mutations (although unlikely, given that selection was no longer than ~50 generations). Further, accurate predictions of the effects of various mutations are almost impossible to achieve and the pleiotropic effects can be complex and far reaching (KNIGHT *et al.* 2006). For a start, WspF has two functional domains [in *E. coli*, the C-terminal domain of CheB functions constitutively in the absence of the input domain (LUPAS and STOCK 1989)]; mutations could therefore have effects ranging from impacts on the flow of phosphoryl groups to activity of the methylesterase domain. Even in the case of frameshift mutations, it is not possible to rule out polymerase slippage, which might be sufficiently significant to ensure that at least some functionally active protein is produced. In addition, there might be subtle transcriptional effects; indeed, transcriptional effects on *wss* [due to changes in the phosphorylation status of WspR (SPIERS *et al.* 2002)] could further complicate understanding. Perhaps more than anything else, this result serves to show how little we do know about the relationship among genotype, phenotype, and fitness.

The microbiological significance of Wsp: The biological function and ecological significance of Wsp in the ancestral genotype remains a mystery. While this work shows that Wsp regulates the production of acetylated cellulose polymer, all our insights stem from the analysis

of variants in which ordinary functioning of the Wsp pathway has been disrupted. Given our understanding of the regulation of the Wsp pathway—complete with integral feedback control—it is safe to assume that the WS phenotype is never achieved by the ancestral genotype, no matter how activated the pathway. The WS phenotype is an altogether novel state achievable only when the Wsp regulation is compromised by mutations that inactivate the integral feedback control. Our hunch is that Wsp, in response to signals unknown, controls the activity of adhesive components in such a way as to effect a kind of colony-based mobility. However, even here we cannot be sure, given that conclusions are largely based on the analysis of constitutive mutants. For example, the primary function of WspR may not be to activate the cellulose synthase enzymes: such activation in WS may be an indirect consequence of a cellular abundance of c-di-GMP. Indeed, the recent discovery of alternate mutation routes to WS—both involving constitutive activation of di-guanylate cyclases that also cause overproduction of the acetylated cellulose polymer—lends weight to this possibility (M. J. McDonald, S. M. Gehrig and P. B. Rainey, unpublished results).

Conclusion: As others and we have previously commented, adaptive evolution, even in supposedly simple experimental microbial populations, is extraordinarily complex. The fitness effects of the simplest possible mutations can be profound and the capacity of "simple" genomes to generate phenotypic novelty can be quite beyond expectation. Unraveling this complexity is a necessary part of any attempt to shed mechanistic light on the evolutionary process and is necessary to ground theory that ties together the connections among genotype, phenotype, and fitness through evolutionary time. Indeed, the more we discover, the greater the parallels between the kinds of genetic changes and their phenotypic effects observed here and the kinds of events thought to have been significant for the evolution of complex metazoan body plans. This appears to be so, even though our analysis at this stage has been confined to genotypes accessible through minimal (single-step) mutational events from the ancestral type.

Finally, with more than passing interest, we note the recent detection of spontaneous *wspF* mutations in *P. aeruginosa* populations colonizing the airways of cystic fibrosis patients (Smith *et al.* 2006). Given that nonpolar transposon insertions in *wspF* of *P. aeruginosa* PAO1 generate WS (D'Argenio *et al.* 2002)—albeit WS that are not cellulose based—it appears that wrinkly spreaders have an evolutionary significance beyond the bottle.

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