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# MINIREVIEW

# Two-Component Signal Transduction as a Target for Microbial Anti-Infective Therapy

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Bacteria are continually bombarded by a multitude of chemicals from their environment, some of which may serve as potential sources of carbon, nitrogen, and energy, while others may act as poisons of their metabolic and regulatory processes. This mix of environmental signals and insults is highly variable and most certainly locale dependent. The potential for an organism to grow and divide within any locale or ecological niche is determined genetically by its repertoire of genes and by its capacity to induce new gene expression to cope with new environments. Often a bacterial infection results from an organism moving from an environment where its presence is benign (e.g., the gastrointestinal system) to another environment where it poses a serious problem (e.g., the urinary tract). This movement between ecological niches requires that the organism "sense" its presence in the new environment and "respond" by expressing new genetic information to permit it to occupy and grow within it. Success in this endeavor is the result of the sum of incremental genetic responses to the new environment of the host.

## HOW MICROORGANISMS SEE AND RESPOND TO THEIR ENVIRONMENT

Microorganisms sense a large number of environmental signals and process much of this information using two-component signal transduction systems (55, 73). These systems combine signal recognition, signal transduction, and gene activation in a two-protein system. Two-component systems consist of a sensor histidine kinase and a response regulator (Fig. 1). The sensor kinase is the primary signal transduction protein that interacts directly with a signal ligand or with a receptor that binds to the signal ligand. Binding of the ligand induces an autophosphorylation reaction in which the γ-phosphate of ATP is transferred to a histidine residue on the kinase. The signal information now exists as a phosphoryl moiety poised to be transferred to a response regulator.

Each sensor kinase is mated to a response regulator protein that carries out the action, usually activation of specific gene transcription, to respond to the signal. The interaction of phosphorylated sensor kinase and its cognate response regulator results in a phosphotransferase reaction in which the phosphoryl group is transferred from the sensor kinase to an aspartate residue on the response regulator (Fig. 1). In general, response regulator transcription factors consist of two major domains, the response regulator and a DNA-binding domain. Phosphorylation of the response regulator domain activates the transcription-regulating functions of the DNA-binding domain. The genes that this protein controls are determined by the specificity of the DNA-binding domain. Response regulators are the "on-off" switch in this system depending on their state of phosphorylation.

The phosphorylated state, or "on" position, of response regulators is regulated in several ways. Many of the kinases that phosphorylate the response regulators may also dephosphorylate them. The presence or absence of a signal could influence either phosphorylation or dephosphorylation depending on the kinase. Thus, the ratio of "on" to "off" switches can be rapidly adjusted to reflect the input signal level. The phosphorylated residue of response regulators is an aspartate, and the phosphoryl-aspartate mixed anhydride bond is susceptible to nonenzymatic hydrolysis. Some or all of the response regulators may possess autophosphatase activity in addition (69).

Phosphoryl-aspartate phosphatases with exquisite specificity exist for some phosphorylated response regulators (57–59). These phosphatases are products of genes regulated by signals other than those that regulate the kinases. Such an arrangement allows more than one signal to influence the phosphorylation state of a response regulator. This assumes importance for response regulators that control cellular systems (e.g., differentiation and pathogenesis) in which many and varied signals must influence the outcome.

## NETWORKING

At this point, it is important to realize that response regulators are subject to regulation from a variety of sources and the phosphorylated (active) state of these proteins may be subject to dephosphorylation reactions that return it to an inactive state. A higher level of control on the activity of two-component systems also exists, and this higher level of control is woven in the fabric of overall cellular control of genetic responses. Viewed in a whole-cell context, a given two-component system may depend on the functioning of another regulatory system or systems for its own activity. This networking of systems involves a higher order of complexity than can be fully developed in this minireview. However, some appreciation of this intricacy may be gained from the model systems depicted in Fig. 2.

Three two-component systems are considered when a signal activates a kinase that, in turn, activates the response regulator necessary for a certain discrete set of genes to be expressed. One of the genes activated by signal A is necessary for pro-

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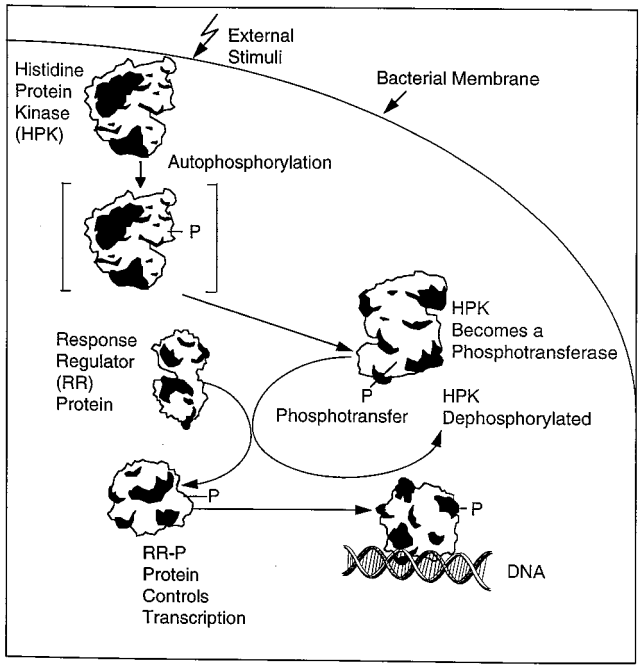


FIG. 1. Typical two-component signal transduction system.

ducing signal B. Thus, the genes induced by signal B are dependent on signal A for their expression. Furthermore, the B pathway regulates the A pathway because one of the genes expressed under signal B control produces phosphatase B that deactivates the signal A pathway. This reciprocal regulation between two-component pathways ensures that the B-regulated genes are expressed after the A-regulated genes, establishing a temporal order of expression. The phosphatase induced as a component of the B-regulated genes deactivates the A pathway regardless of the presence of signal A. This establishes the primacy of the B pathway and also serves to limit its time of expression. Signal C also induces a phosphatase (phosphatase C) whose role is to prevent the B pathway from functioning. In this case, the A pathway continues to function as long as the C pathway is active. Phosphatases might also be induced as components of the A pathway to ensure that the B or C pathway does not function. Some genes may require more than one two-component regulator to express and thus establish a codependence between the pathways. These phosphatase interactions between two-component signal transduction pathways were discovered as part of the cellular control of development in *Bacillus subtilis* (57) but are likely to be found in many organisms with complex signal transduction networks.

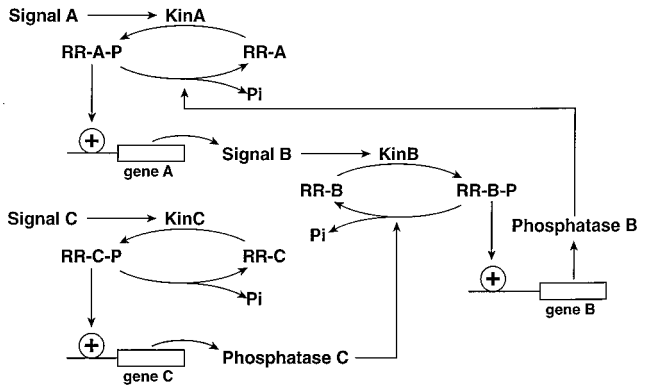


FIG. 2. Simple example of some principles of networking in two-component signal transduction.

This brief discussion of networking has not taken into account many other factors that are required for gene expression and that are also subject to regulation. Catabolite repression and other global regulators regulate genes across signal transduction pathways. Sigma factors group genes into distinct regulated units. Stresses of many kinds (heat shock, cold shock, etc.) induce regulons of genes and repress others. Any given gene may be targeted for expression by one or several regulators. The number of possibilities or combinations of regulators is enormous.

Networking is only part of the answer to overall cellular control in complex responses to environment stimuli such as those found in pathogenesis or differentiation in bacteria. Both of these cellular responses result from the activation of a large number of genes under the control of several different transcription regulators. Only a few of the gene products from any given regulator may be necessary for pathogenesis or differentiation, and they may arise from different regulators. Thus, the entire gamut of responses may be required to produce a full complement of proteins necessary for the process, while most of the proteins produced are superfluous. Two-component regulation of many cellular processes may represent the only common element among unrelated processes and may provide a means for generalized cellular shutdown by a broad-spectrum inhibitor.

In this minireview, we focus on the role of two-component regulatory systems in virulence and growth and argue that their essential role and universality in those mechanisms make such systems attractive targets for anti-infective therapy.

## VIRULENCE FACTORS IN THE PROCESS OF PATHOGENICITY

The production of disease due to bacterial infection requires temporal and coordinated expression of a series of genes that allow the prospective pathogen to adapt to the hostile environment in the host. The expression of these genes contributes to the virulence of these pathogens, and such genes encode products frequently termed "virulence factors." Such products could include enzymes required to metabolize complex proteins and glycoproteins found in connective tissues or blood, bacterial toxins, cell surface proteins that mediate bacterial attachment, cell surface carbohydrates and proteins that protect a bacterium, and hydrolytic enzymes that may contribute to the pathogenicity of the bacterium. Bacterial surface structures that permit both attachment and entry into epithelial cells have been characterized, and pathogenic bacteria may use any of a number of protein-cleaving factors to infect the host through any mucosal surface (gastrointestinal, respiratory, etc.) (35, 37, 56, 78). In addition, some bacteria (e.g., group A streptococci) contain cell wall structures such as lipoteichoic acid and/or M proteins that facilitate attachment and infection, whereas others (e.g., *Escherichia coli*) contain capsular polysaccharides (K antigens) and adhesions to accomplish homologous functions (25, 44, 61, 81). The factors that contribute to virulence and the mechanisms of virulence regulation are as yet not fully understood; however, significant progress in our understanding of the genetics, biochemistry, and molecular biology of virulence has been achieved during the last decade (7, 32, 35, 56).

Factors that encode for, regulate, or facilitate the transfer of

antibacterial or antibiotic resistance may also contribute to the bacterium's shift to the pathogenic state. From the standpoint of the bacterium, virulence factors contribute to the ability of the microorganism to survive and grow at the site of infection and to its pathogenicity, and thus, in an ecological sense, virulence factors contribute to how well a microorganism propagates in a mammalian host. By using this perspective, the definition of virulence factors should be expanded to include the products of genes that may not necessarily contribute to the production of clinical signs and/or lesions but contribute to the array of products required to be able to cope with the metabolic substrates (or lack of substrates) provided in the mammalian habitat.

Successful infection involves a chain of events in which a pathogen may have to adhere and/or break through the barriers of organized tissues, evade the phagocytic and immune response defenses of the host, grow under conditions in which particular nutrients are limiting, and combat an overall hostile environment. Thus, for pathogens such as *Salmonella typhimurium* that survive within particular cellular compartments, e.g., phagosomes, the induction of particular metabolic genes becomes essential for survival in the host (4, 24). Furthermore, in the course of establishing an infection many pathogens invading a host may have to adapt to several new environmental conditions. Because of this the invasion of a host cell very likely requires a series of regulatory genes functioning in a temporal order so that the bacterium can cope with changing environments as infection progresses (30). Therefore, regulator genes that control the expression of virulence factors essential for the pathogen's survival may be considered "virulence factors."

## VIRULENCE REGULATION BY TWO-COMPONENT SIGNAL TRANSDUCTION SYSTEMS

It is now clear that bacterial pathogens are very adept at resolving host-environment-pathogen interactions by evolving virulence factors and regulation systems that allow them to survive in many hostile environments. Impairment of one or more of these virulence factors by mutation, antibody neutralization, or chemical inhibition can be the determining factor in tipping the outcome of the infection favorably toward the host. As a consequence, we are gaining an appreciation for the potential usefulness of bacterial virulence factors as new targets for therapeutic intervention against antimicrobial agent-resistant pathogens. Two-component signal transduction systems are the only common regulatory elements shared by a wide range of virulence systems, raising the possibility that a broad-spectrum inhibitor of such elements may suppress virulence in a variety of microorganisms (32, 54).

Two-component systems are ubiquitous in bacteria, and in all free-living species thus far tested there are multiple two-component systems. Two-component system proteins of different species of bacteria share common sequence motifs, particularly among amino acid residues located near or around the active site (73) and, in the case of response regulators, share a high degree of structural homology (23). More than 100 two-component systems have been reported in bacteria, in lower eukaryotes including the yeast *Saccharomyces cerevisiae* (49), the slime mold *Dictyostelium discoideum* (71, 85), and the fungus *Neurospora crassa* (2), and in the plant *Arabidopsis thaliana* (14). No systems of this type have been found in mammals, despite concerted searches in several laboratories. Two-component systems control many of the virulence factors required for bacteria to survive in the foreign host and "essential" genes in some bacteria (38, 63). The yeast and fungal two-component systems regulate responses to osmolarity; mutants are osmosensitive and are likely to be impaired as pathogens. A representative list of two-component systems involved in bacterial virulence processes is presented in Table 1.

## VIRULENCE FACTORS THAT ARE ALSO ANTIBACTERIAL RESISTANCE FACTORS

One of the more fascinating aspects of two-component system control is the role of these systems in resistance to certain antibacterial agents. The three specific examples, presented in Table 2, are in bacteria that are becoming clinical problems because of the resistance. Although a two-component system is used in each resistance system for regulation of its resistance gene(s), the actual involvement is different in each system.

Vancomycin resistance in staphylococci and enterococci has the potential of becoming a serious clinical problem (12, 13, 53, 60). The presence of the resistance genes on a transposon responsible for the VanA-type phenotype in enterococci provides a mechanism for resistance to spread across species barriers (5, 22). The transposon contains genes coding for mobility as well as resistance (5). Resistance is regulated by the sensor kinase VanS and its response regulator VanR in reaction to some as yet unknown signal (36). VanR is a transcription activator of the genes responsible for synthesis of the depsipeptide D-alanyl-D-lactate that is incorporated into the peptidoglycan and gives rise to antibiotic resistance (5). VanB-type vancomycin resistance gene expression is also affected by VanS-VanR (22).

Tetracycline resistance in *Bacteroides* strains is mediated by a conjugative transposon. The conjugal frequency is increased by tetracycline through the *rte* two-component system (64). *rteA* encodes a sensor kinase closely related to BvgS of *B. pertussis* (8). The *rteB* gene codes for a response regulator similar to NtrC of *E. coli*, and its transcription-activating function is thought to require the sigma-54 counterpart of *Bacteroides*. Based on structural homology, the RteA-RteB system is likely to proceed through a phosphorelay in which different portions of RteA correspond to those of BvgS. An antibacterial agent directed toward two-component systems should prevent the increase of tetracycline resistance in the species during tetracycline therapy.

Another variation on the theme of the two-component system control is found in *Streptococcus pneumoniae*, in which the penicillin-binding protein mediating high-level penicillin resistance is under the control of two-component regulation. In a complex and not fully understood system, the observation has been made that the two-component system in *S. pneumoniae*, *ciaH* and *ciaR* (encoding a kinase and a response regulator, respectively), is involved in both competence and penicillin susceptibility (34, 93). In the process of selecting for cefotaxime resistance in *S. pneumoniae*, the unpredicted phenotype of loss of transformability and high-level cefotaxime resistance was found in four mutants (34, 93). In such mutants, the mutation responsible for both phenotypes was mapped to *ciaH*, which encodes the putative sensor kinase protein of the two-component system for competence in *S. pneumoniae*. This observation has similarities to the signal transduction control over the VanH phenotype, and both systems are coupled to the biosynthesis of the cell wall.

## NATURAL INHIBITORS

Only a few natural inhibitors of two-component regulatory systems have been reported. Unsaturated fatty acids are noncompetitive inhibitors of ATP-dependent autophosphorylation of the histidine protein kinase KinA involved in the regulation

TABLE 1. Two-component system-controlled virulence and resistance factors

| Factor and gene(s) | Microorganism | Component | Reference(s) |
|---|---|---|---|
| Surface antigen | | | |
| *algR-agrD* | *Pseudomonas aeruginosa* | Alginate capsule | 17, 31 |
| *cpxR-cpxA* | *Escherichia coli* | Capsule | 20 |
| *rmpA2* | *Klebsiella pneumoniae* | Capsule production | 84 |
| *rcsB-rcsC* | *Escherichia coli* | Capsule | 43 |
| *viaA-viaB* | *Citrobacter freudii* | Vi antigen expression | 41 |
| Virulence factors | | | |
| *agrA-agrC* | *Staphylococcus aureus* | Global virulence regulator | 54 |
| *bvgA-bvgS* | *Bordetella pertussis* | Virulence | 1, 8 |
| *cheY-cheA-cheB* | *Salmonella typhimurium* | Motility | 11, 82 |
| *finG-finN* | *Salmonella typhimurium* | Flagella | 29, 42 |
| *hrpB* | *Pseudomonas solanacearum* | Pathogenicity | 80 |
| *ipaA-ipaB-ipaD* | *Shigella flexneri* | Mammalian entry | 92 |
| *mga* | Group A streptococci | M-protein virulence | 50 |
| *mxiD* | *Shigella flexneri* | OMPs[a] for secretion of *Ipa* invasion | 3 |
| *ompR-envZ* | *Salmonella, Escherichia coli, Shigella* | Virulence OMPs | 9, 86 |
| *phoQ-phoR* | *Salmonella typhimurium* | Virulence | 24, 30 |
| *pilA-pilB* | *Neisseria gonorrhea* | Pilus production | 6 |
| *pilS-pilR* | *Pseudomonas aeruginosa* | Fimbria and pilus formation | 40 |
| *pmrA* | *Salmonella typhimurium* | Virulence | 65 |
| *prfA* | *Listeria monocytogenes* | Virulence | 89 |
| *pilG* | *Pseudomonas aeruginosa* | Twitching motility | 16 |
| *spv* | *Salmonella* | Virulence | 33 |
| *vacB* | *Shigella flexneri* | Virulence plasmid | 77 |
| Exotoxins, enzymes | | | |
| *exo* | *Pseudomonas aeruginosa* | Exoenzymes | 26 |
| *ntrA-ntrC* | *Klebsiella pneumoniae* | Urease production | 15 |
| *toxS-toxR* | *Vibrio cholera, Vibrio parahemolyticus* | Virulence (toxin, pili, hemolysin) | 18 |
| *virR* | *Clostridium perfringens* | Regulation of collagenase, perfringolysis O, and hemagglutini | 72 |
| Resistance factor | | | |
| *rprX-rprY* | *Bacteroides fragilis* | Porin-mediated tetracycline resistance | 64 |
| *vanR-vanS* | *Enterococcus faecalis* | Vancomycin resistance | 5 |

[a] OMPs, outer membrane proteins.

of sporulation in *B. subtilis* (75). Oleic acid inhibits the formation of KinA-phosphate in a concentration-dependent manner in the presence or absence of the cognate response regulator. Oleic acid does not inhibit bacterial growth.

Two natural inhibitors that affect the activity of a two-component system which allows *Agrobacterium tumefaciens* to infect plant wounds and induce crown gall tumors in dicotyledonous plants were identified (45). Acetosyringone and/or other phenolic compounds produced by wounded tobacco cells are the environmental stimuli that activate this two-component system (46, 90). Bromoacetosyringone completely inhibits expression of genes regulated by the two-component system at concentrations that do not affect *A. tumefaciens* growth (51). Bromoacetosyringone binds irreversibly and does not affect other inducible genes.

TABLE 2. Resistance problems mediated by two-component system regulation

| Microorganism | Disease | Resistance problems |
|---|---|---|
| *Enterococci* | Systemic infections | Vancomycin, teicoplanin |
| *Streptococcus pneumoniae* | Respiratory infections | Penicillin, cephalosporins |
| *Bacteroides* | Abdominal infections | Tetracycline |

## SYNTHETIC INHIBITORS

Synthetic inhibitors of two-component systems that regulate the expression of virulence factors in bacteria pathogenic for humans have been reported for the *P. aeruginosa* two-component system that controls the alginate biosynthetic pathway (67). Alginate is synthesized by *P. aeruginosa* and forms a protective exopolysaccharide coat that plays an important role in the pathogenesis of this microorganism in cystic fibrosis patients (66). The synthesis of alginate in patients' lungs is positively controlled by a two-component system, AlgR2-AlgR1 (67). Several compounds were identified as potent inhibitors of *algD* promoter activity (Fig. 3). Compounds 1 and 2 inhibited the autophosphorylation of AlgR2, the autophosphatase of AlgR2, and the phosphotransfer of the phosphate to AlgR1, but they did not inhibit the binding of AlgR1 to the *algD* promoter (67). Compounds 3 and 4 did not inhibit the autophosphorylation, the autophosphatase, or the phosphotransferase of AlgR2, but they did inhibit the binding of AlgR1 to the *algD* promoter. Inhibitors such as these are not antibacterial agents but have the potential for use as therapy in combination with an antibacterial agent. In theory, an inhibitor of the alginate two-component system would render the bacterium susceptible to bactericidal antibacterial agents and to phagocytosis by weakening the protective alginate barrier.

Recent reports have demonstrated the application of two-

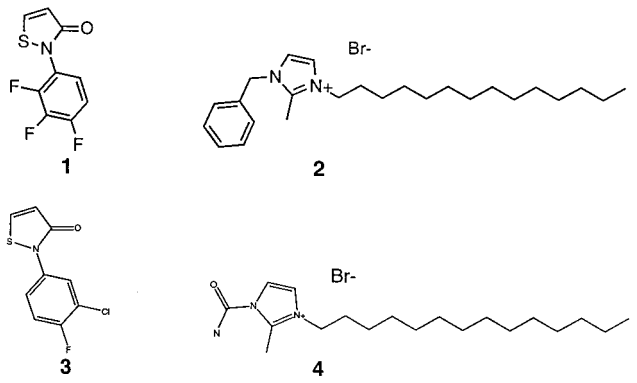


FIG. 3. Chemical structures of the antialginate two-component kinase inhibitors. More than 95% of *algD* promoter inhibition activity was observed for inhibitors 1 and 2 at 2 to 2.5 μg/ml. Compounds 1 and 3 also inhibit AlgR1, whereas compounds 2 and 4 do not, but compounds 2 and 4 do inhibit other kinases (66, 67).

component system inhibitors as antibacterial agents. Domagala et al. (19) reported the structure-activity relationship of two-component system inhibitors of gram-positive organisms for several series of diphenol-methane compounds that possess antibacterial activity (Fig. 4). Structures PD-164592 and PD-163892 are representative chemotypes in this series. These compounds were identified from high-throughput screening for inhibitors against the $NR_{II}$ kinase. The MICs of these compounds for gram-positive bacteria (*B. subtilis*, *Staphylococcus aureus*, and *Streptococcus pyogenes*) were found to be from 1 to 4 μg/ml. The MICs for methicillin-resistant *S. aureus* were as low as 4 μg/ml, and at 2 μg/ml these compounds were active against enterococci. Research indicates that the phenol nucleus is essential for inhibitory activity (19). Little or no activity against gram-negative bacteria was observed for the diphenolmethanes. Overall, there was a 85 to 90% correlation between the 50% inhibitory concentration for the inhibition of $NR_{II}$ kinase activity and the MICs for gram-positive bacteria (19).

A series of reports has also emerged from researchers at the R. W. Johnson Pharmaceutical Research Institute. Five chemotypes (Fig. 5) that possess two-component system inhibitory activity and that result in antibacterial activity have been reported (27, 47, 48, 52, 62, 79, 87, 88, 91). These compounds are primarily active against gram-positive bacteria and demonstrated a mechanism-based inhibition of two-component systems in reporter gene systems. Triphenylalkyl derivatives (88), cyclohexenes (91), salicylanilides (48), benzoxazines (27), and bisphenols (biphenol methanes) (87) were reported as inhibitors, with supportive data including MICs, target-based data demonstrating in vitro activity against KinA::SpoOF and/or $NR_{II}/NR_I$ (as well as other two-component systems), killing curves, compound binding data, structural data (62), and in vivo data (52).

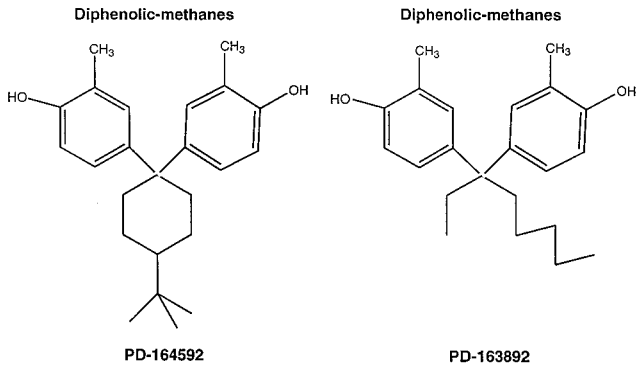


FIG. 4. Prototypical structures of diphenolic methanes with $NR_{II}$ inhibitory activity and weak activity against gram-positive bacteria (19).

RWJ-49445, a representative of the cyclohexene series (79), has MICs of 1 to 4 μg/ml for gram-positive bacteria and is rapidly bactericidal. The salicylanilides, based on the antihelminthic drug closental, inhibited both KinA::SpoOF and $NR_{II}$:$NR_I$ and possessed potent antibacterial activity in vitro. MICs were in the range of 0.5 to 4 μg/ml, with rapid bacterial killing. The benzoxazines, represented by RWJ-63138 and RWJ-63093, were also shown to be potent antibacterial agents (27) but were limited in their in vivo activity due to the serum-binding effects in vivo (47, 52). The triphenylalkyl derivative series (88), closely related to the cyclohexenes and the bisphenols (87), were also potent agents against gram-positive bacteria. Several series were reported as having in molecularly engineered reporter gene systems in vitro "proof-of-principle" activity, indicating that they have activity against a specific two-component system in the whole bacterium (48). For example, the salicylamide series possessed activity in the so-called Taz-1 assay (which specifically measures OmpR activation), indicating a mechanism-specific effect at sub-MIC in *E. coli* (48). Additionally, the salicylamide series inhibited in vitro the van-Luc assay system, a modification of the *vanR-vanS* system in *Euterococcus faecium*, which is an indication of specific two-component system inhibition at sub-MICs in the whole bacterium (48).

It is important to remember that while all of these compounds inhibit both bacterial growth and two-component systems, no absolute proof that growth inhibition is the direct consequence of inhibition of two-component systems has been presented. Furthermore, the possibility that any of these compounds will reach the clinic requires an assessment of their toxicological properties.

## POSSIBLE SITES OF TWO-COMPONENT SYSTEM INHIBITION

Recent reviews (10, 21, 28, 68, 70, 76) have described the importance to bacteria of networks of intracellular signaling in the bacterium in response to its environment. The interruption of these signals may lead to the interruption of virulence and/or a decrease in the levels of bacterial virulence factors. Such targets may offer the opportunity for a totally new class of antibacterial agents (7, 39).

The possible sites of two-component system intervention in situ include the autophosphorylation signal, autophosphorylation of the sensor-kinase, interaction of the sensor~P:: response regulator (RR), phosphotransfer of sensor-kinase~P to RR, dephosphorylation of the sensor-kinase~P, and binding to the regulated gene promoter (67, 74). If a molecule targets the active sites of two-component systems that are common to all, then inhibition of multiple two-component systems in bacteria may be possible in a single bacterium. With these two-component arrays having high degrees of homology across gram-positive and gram-negative bacterial species (83), a single two-component inhibitor may be broad spectrum if a single agent targets a common site. An agent that specifically inhibits the site of interaction between a phosphorylated response regulator and the promoter of the gene that is controlled should result in a selective inhibition of just that bacterium. Thus, from a theoretical standpoint, inhibitors could act from the extremes of species-specific to broad-spectrum antibacterial agents.

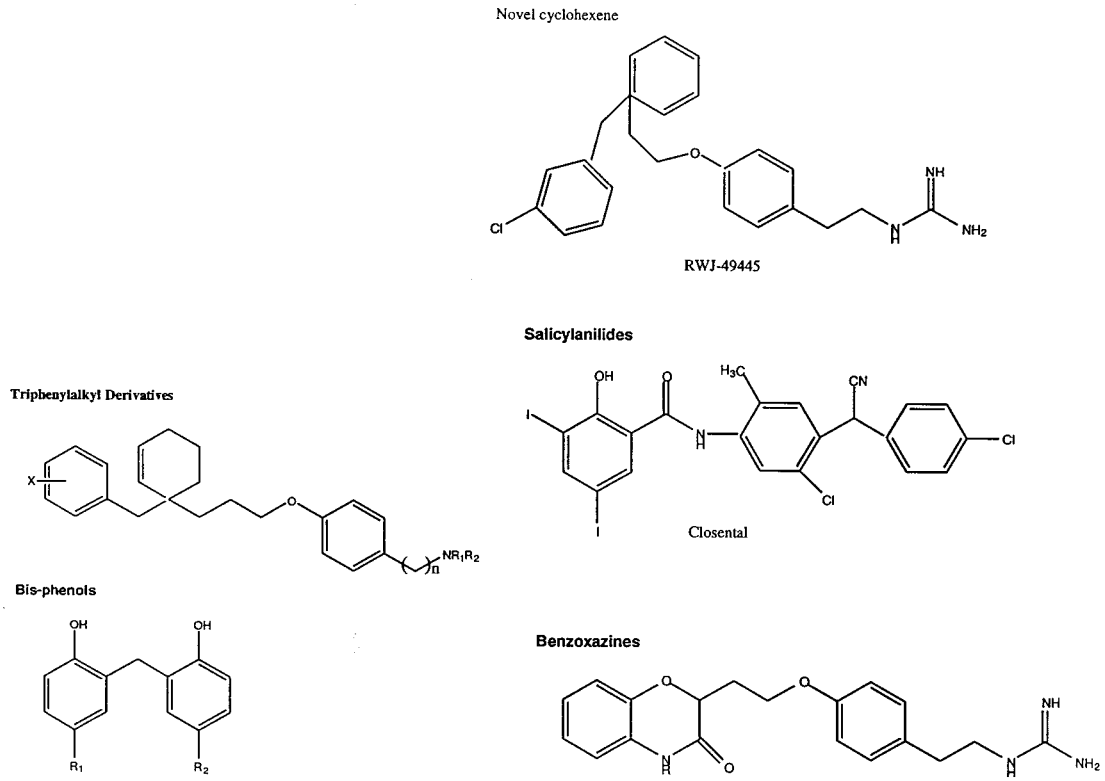


FIG. 5. Prototypical structures from the efforts of the R. W. Johnson Pharmaceutical Research Institute. These five series represent the reported inhibitors in a variety of in vitro, microbiological, and in vivo test systems (27, 47, 48, 52, 62, 79, 87, 88, 91).

## PERSPECTIVE

Pathogenicity is the result of complex genetic regulation of a surfeit of seemingly unrelated responses to environmental influences. The thesis proposed here is that interference with the regulation of such processes by targeting a common element of regulation such as two-component systems is likely to prevent pathogenesis in a host regardless of whether the inhibitor affects growth ex hominis. While this notion may inflame some readers, if this minireview makes a few people appreciate that complex cellular regulation in processes such as virulence in microorganisms is the sum of seemingly inconsequential parts, it will have succeeded.

## ACKNOWLEDGMENTS

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