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Characterization of Combinatorial Patterns Generated by Multiple Two-Component Sensors in *E. coli* that Respond to many Stimuli

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Abstract

Two-component systems enable bacteria to sense changes in their environment and adjust gene expression in response. Multiple two-component systems could function as a combinatorial sensor to discriminate environmental conditions. A combinatorial sensor is composed of a set of sensors that are non-specifically activated to different magnitudes by many stimuli, such that their collective activity pattern defines the signal. Using promoter reporters and flow cytometry, we measured the response of three two-component systems in *Escherichia coli* that have been previously reported to respond to many environmental stimuli (EnvZ/OmpR, CpxA/CpxR, and RcsC/RcsD/RcsB). A chemical library was screened for the ability to activate the sensors and 13 inducers were identified that produce different patterns of sensor activity. The activities of the three systems are uncorrelated with each other and the osmolarity of the inducing media. Five of the seven possible non-trivial patterns generated by three sensors are observed. This data demonstrate one mechanism by which bacteria are able to use a limited set of sensors to identify a diverse set of compounds and environmental conditions.

Keywords

systems biology; synthetic biology; membrane fluidity; butanol; isobutanol; biofuel; biodiesel; stress response

Introduction

Two-component systems are a common sensing motif in bacteria that connect environmental signals to changes in gene expression (Beier and Gross, 2006; Majdalani and Gottesman, 2005). Bacteria have multiple two-component systems, many of which operate simultaneously (*Escherichia coli* has ~32) (Hoch and Silhavy, 1995). In some cases, the sensor is specific, responding to a single stimulus and regulating a limited number of genes that are specific to that stimulus (Hong et al., 2008). Other two-component systems are stimulated by multiple conditions and regulate many genes of diverse function (Prohinar et al., 2002; Rampersaud and Inouye, 1991; Thomas and Booth, 1992). The most general

sensors are stimulated by many chemically distinct compounds and are often assigned a function of sensing osmolarity (Alphen and Lugtenberg, 1977; Jubelin et al., 2005; Sledjeski and Gottesman, 1996).

Two-component sensors that respond to many inputs could participate in a combinatorial sensor. A combinatorial sensor is made up of a set of sensors, each of which is activated by many stimuli and it is the pattern of their activation that defines the signal (Dodd et al., 1991; Littarru, 2007; Schmiedeskamp, 2001; Turner and Magan, 2004). The mammalian olfactory system is an advanced example of a combinatorial sensor. The human nose has ~400 different chemoreceptors, yet it can recognize ~10,000 different odorants (Ache and Young, 2005; Bar-Cohen, 2006). This is accomplished via a combinatorial recognition of odorants. The nose does not have a single receptor for every odorant; rather, there is a set of receptors that bind non-specifically to many chemicals with different affinities (Lewis, 2004). A chemical produces a signature pattern of receptor activities, which is processed by the brain to identify the odor (Lauren, 2002). There are two advantages of such a sensor. First, a small number of receptors can respond to a large number of chemicals. Second, the system can be trained to respond to new stimuli without the addition of new receptors. This is achieved by responding to a new activity pattern.

Combinatorial sensing with two-component systems could be an efficient strategy bacteria have evolved to sense multiple inputs with a limited set of sensors. Following this analogy, multiple two-component systems would respond to different inputs, and the downstream regulatory network would interpret the activity pattern. For combinatorial sensing to take place, the sensors should: (1) respond to multiple stimuli, (2) be independent, such that different patterns are generated in response to the stimuli, (3) have a regulatory mechanism by which the pattern is recognized. This manuscript focuses on the first two of these requirements, where we have identified different patterns produced by chemical stimuli, and quantify the independence of the information provided by the sensors. Regarding the third requirement, it is common for the activities of multiple two-component systems to be integrated on the level of the phosphorelay or to co-regulate a promoter (Hoch and Silhavy, 1995).

To characterize combinatorial sensing, it is useful to work with sensors that respond to the most stimuli as it is easier to identify conditions that produce different patterns of sensor activity. The *E. coli* two-component systems EnvZ/OmpR (Hall and Silhavy, 1981), CpxAR (Mc Ewen and Silverman, 1980), and RcsCDB (Gottesman et al., 1985) each respond to many stimuli, and some stimuli activate multiple sensors (Fig. 1A, with references listed in the SI). The regulons of the three sensors overlap and they regulate diverse cellular processes, such as pathogenicity, flagella synthesis, and biofilm formation (Fig. 1B and Table S1 and S2) (Arricau et al., 2002; Jubelin et al., 2005; Kim and Falkow, 2004; Oshima et al., 2002; Shin and Park, 1995). EnvZ/OmpR have been shown to alter the expression of outer membrane porins ompC, and ompF in response to changes in osmotic pressure (Aiba et al., 1989). However, doubt has been expressed as to its literal role as an osmosensor (Batchelor and Goulian, 2003; Batchelor et al., 2005; Price and Raivio, 2009; Prohinar et al., 2002; Rampersaud and Inouye, 1991). CpxAR responds to cell envelope stress, and regulates genes involved in periplasmic protein folding and degradation. Multiple signals stimulate CpxA through periplasmic proteins (Danese and Silhavy, 1998; DiGiuseppe and Silhavy, 2003; Nakayama and Watanabe, 1995). RcsCDB is activated by conditions that lead to envelope stress, such as contact with surfaces and the over-expression of periplasmic proteins (Majdalani and Gottesman, 2005). Like CpxA, RcsC senses some signals through periplasmic mediators (Castanie-Cornet et al., 2006; Laubacher and Ades, 2008).

In this manuscript, we have quantified the patterns of activity produced by the EnvZ/OmpR, CpxAR, and RcsCDB sensors when cells are stimulated with different chemicals. First, we constructed reporter plasmid that contains transcriptional fusions between promoters that respond to each two-component system and a fluorescent reporter. The patterns of sensor activity were determined for a set of inducers, including chemicals and pH changes. This induction data was used to quantify the degree to which each sensor responds non-specifically to inputs, as well the independence of the activities between sensors. By visualizing the patterns of sensor activity, we determined the diversity of patterns that are generated as well as how different chemicals cluster in pattern space. Combinatorial sensing represents a new paradigm for understanding how the integration of multiple general sensors improves specificity and this may lead to new approaches for the forward engineering of genetic sensors.

Materials and Methods

Strains, Plasmids, and Media

Strains *E. coli* BW28357 [Δ (araD-araB)567, Δ lacZ4787(::rrnB-3), l-, Δ (rhaD-rhaB)568, hsdR514], BW29655 (BW28357 Δ envZ/ompR), BW29849 (BW28357 Δ cpxA/cpxR), and BW27870 (BW28357 Δ rcsB) were obtained from the Coli Genetic Stock Center at Yale (Zhou et al., 2003). All promoter reporters are variants of pAC581 (Anderson et al., 2006). This plasmid possesses a p15A origin, chloramphenicol resistance marker, and a codon optimized GFPmut3. GFP expression is under the control of various promoters. *pompC* (2310952...2310802), *pcpxP* (4103753...4103858), and *prprA* (1768254...1768396) were amplified from *E. coli* MG1655 genomic DNA (NC_000913.2). The constitutive control plasmid with promoter J23107 was constructed by inserting annealed primers into pAC581. J23107 is a 35 base pair sequence (5'-tttacggctagctcagccctaggtattatgctagc-3') from a library of constitutive promoters (BBa_J23107, J.C. Anderson, UCB). Assays were performed in M9 minimal media (48 mM Na₂HPO₄, 22 mM KH₂PO₄, 16 mM NH₄Cl, 2 mM MgSO₄, 0.1 mM CaCl₂, and 8.6 mM NaCl, pH 7.4) with 0.4% glucose as the carbon source and 30 μ g/mL chloramphenicol. Cells were plated on LB agar, consisting of 25 g of LB broth (Difco, Franklin Lakes, NJ #244610) and 18 g Bacto agar (Difco #281210) per liter.

The inducer library. The 11 chemicals that were used as inducers are: Acetone (4%; Sigma, St. Louis, MO-650501), Butanol (0.54%; Sigma-B7906), Ethanol (3%; Gold Shield, Chemicals Co., Hayward, CA), Indole (3 mM; Fisher, Waltham, MA-06104), Isobutanol (0.3%; Fluka, St. Louis, MO-82059), KCl (550 mM; Fisher-S77375), Methanol (4.8%; Sigma-34898), NaCl (550 mM; Acros organics, Belgium Germany-327300010), Pentanol (0.3%; Fluka-77597), Phenethyl Alcohol (0.2%; Sigma-P13606), Procaine (17 mM; Sigma-P9879). Each chemical was added to M9 media. Table S3 lists the OD₆₀₀ after 2 h of growth and the osmolarity of M9 when each chemical is added. The complete list of chemicals that did not generate a response >2-fold for at least one sensor is shown in Table S4.

Induction assay. Two effects were observed that influenced the design of the induction assay. First, all of the reporters, especially those for EnvZ/OmpR, became more active upon entry into stationary phase. Second, differences in temperature, growth rate, and media composition impacted induction. This has been observed previously, where certain chemicals only induce these two-component systems under specific growth conditions, such as low temperature or a specific carbon source (Hagiwara et al., 2003). Thus, we measured induction only over logarithmic growth, kept temperature constant by growing in water bath shakers, and grew cells in M9 minimal media, whose composition and pH could be carefully controlled. The growth and induction assay was optimized to reduce day-to-day variation.

Cells were streaked from glycerol stocks onto an LB agar plate and grown for 16 h. A culture was inoculated from this plate and grown for 14 h, and then diluted 100× into fresh media. At OD₆₀₀ = 0.4, cells were again diluted 100× into 150 mL of fresh M9. These cells were grown at 37°C in a 250 mL glass baffled flask in a shaking water bath (Innova 3100, New Brunswick Scientific, Edison, NJ) at 160 rpm. When cells reached OD₆₀₀ = 0.2, the culture was split into six flasks, one uninduced, and the remainder containing the inducers. Each chemical was supplied at the concentration that led to a 50% growth rate defect as measured at the end of the 2 h (Table S3). For pH 4 induction, cultures were added directly to a flask containing 33 µL of 10 M HCl. Similarly, for pH 10 induction, cultures were added to a flask containing 60 µL of 10 M KOH. The volumes of acid and base needed to reach the desired pHs were determined empirically. Aliquots were taken at $t = 0$ min, 15 min, 30 min, 60 min, 90 min, and 120 min. Protein expression was immediately stopped in each sample with kanamycin (a 100 µL sample was diluted five-fold into PBS containing 2 mg/mL kanamycin) and the samples were stored on ice until flow cytometry was performed.

Flow cytometry. Cells were diluted 100× into phosphate-buffered saline (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na₂HPO₄, 0.24 g/L KH₂PO₄) containing 2 mg/mL kanamycin and measurements were made on a BD Biosciences LSRII flow cytometer (courtesy of the Gladstone Research Institute) with laser settings: FSC,578; SSC,335; GFP,607. Each data set consisted of at least 10,000 cells. The FlowJo software package (Tree Star, Inc., Ashland, OR) was used to gate the data based on the FSC-SSC scatter. From this, a histogram of GFP fluorescence was generated and the arithmetic mean GFP fluorescence was calculated. The fold-induction is the ratio of GFP in induced cells to that of uninduced cells at the 2 h time point. Data points are the average of at least two independent experiments with assays run on different days and picked from different colonies.

Osmolality measurements. The chemicals were added at the appropriate concentration to M9 media, and the osmolality was measured using a Wescor vapor pressure osmometer (model 5520). The machine was calibrated with three standards: 1000, 290, and 100 mmol/kg (Optimole OA-010, OA-029, OA-100, Wescor, Logan, UT). A clean test was performed prior to measurements to ensure linearity and accuracy, as described in the Wescor operational manual.

Data analysis. Principal component analysis (PCA) was performed using the multivariate analysis add-on for Microsoft Excel. The input to the analysis is a 13×3 matrix, where each row corresponds to one of the 13 chemicals, and each column corresponds to the fold-induction of one of the three promoters normalized by the maximum induction for each inducer. The output of the PCA is a 13×2 matrix. In this form, each chemical is represented as a point in two dimensions, where each of the axes is a principal component. There is no biological meaning to these axes; rather, this method enables the visualization of the patterns. Each principal component is a linear combination of the three sensors. The contributions of each sensor are: PC1 (0.025, -0.619, 0.785), and PC2 (0.988, -0.103, -0.112), where each element corresponds to EnvZ/OmpR, CpxA/CpxR, and RcsC/RcsD/RcsB, respectively. PC1 and PC2 together capture 85% of the variance of the original dataset.

Clustering and the elliptical boundaries were drawn by eye to encompass inducers that produce similar patterns, with the constraint that the ellipses point to the black dot, which corresponds to non-induction. To generate the trajectories in Figure 5B, chemical concentrations were excluded that did not induce at least one sensor by greater than two-fold. Each concentration pattern was then projected onto the PCA space generated by the 13 chemicals (Fig. 5A). The patterns produced by the concentration changes were not used to calculate the principal components.

Results

Reporter System and Assay Conditions

Three promoter reporters were constructed to measure the induction of each two-component system: EnvZ/OmpR (pompC), CpxAR (pcpxP), and RcsCDB (prprA) (Figs. 2A and S1). To measure the response to a chemical, cells containing a single reporter plasmid were grown in minimal media until early log phase, and split into either a control flask, or a flask containing inducing chemical (Materials and Methods Section). Time points were taken over 2 h, and flow cytometry was performed (Fig. 2A). Because the reporters are based on natural promoters, it is likely that additional factors contribute directly or indirectly to their induction. To test that the promoters require their cognate response regulator for activity in our conditions, we measured the reporter activity in strains where the response regulator has been knocked out (Fig. 2B). In each case, the activity of the promoter dropped considerably and is no longer inducible.

Sensor Induction by the Chemical Library

A set of 38 inducers (chemicals and pH changes) was compiled (Tables S3 and S4), consisting of compounds that are known or predicted to influence these two-component systems. The effect of each chemical on the three sensor reporters was assayed. The end points of the time course were used to generate the pattern of sensor activities for each inducer. The fold-induction was calculated between the induced and uninduced cells at the end of the time course. Of the 38 initial conditions, a subset of 13 was identified where at least one reporter was induced greater than two-fold and there was not a non-specific increase in fluorescence due to toxicity or growth inhibition (Fig. S5).

Each chemical induces the reporters with differing magnitudes and the rank order of induction changes (Fig. 3A). The top five inducers for EnvZ/OmpR and CpxAR include alcohols, indole, and pH extremes. These seemingly do not have a common mode of action: alcohol is known to fluidize the membrane, indole perturbs the outer membrane, and pH affects levels of both periplasmic and envelope proteins (Dombek and Ingram, 1984b; Stancik et al., 2002). RcsCDB is strongly induced by alcohols and salt—two stresses that have opposite effects on membrane fluidity (Beney et al., 2004; Dombek and Ingram, 1984b). Because each system is activated by many diverse stimuli, the sensors are general and thus, they meet the first requirement for a combinatorial sensor.

The Sensors are Independent and Uncorrelated With Osmolarity

A requirement for a combinatorial sensor is that each individual sensor provides unique information about the stimulus. If the activities of the sensors are highly correlated, then the combination of sensors is unable to provide more information. Figure 3B shows the correlation between the promoter reporters for the set of inducing conditions. There is no correlation between RcsCDB and EnvZ/OmpR or CpxAR. The data is more correlated between CpxAR and EnvZ/OmpR ($R^2 = 0.323$). In all three graphs, there are examples of chemicals that induce one system but not the other. For example, pH5 induced EnvZ/OmpR but not CpxAR, and phenethyl alcohol induced CpxAR strongly, but had less of an effect on EnvZ/OmpR. The lack of correlation between the sensor activities implies that they are responding to different aspects of the chemical stresses.

EnvZ is often referred to as an “osmosensor,” and some reports have implied that the other sensors could be responding to osmolarity (Arricau et al., 2002; Prigent-Combaret et al., 2001). To test this, we measured the osmolarity of the media for each of the inducing conditions (Fig. 3C). There is no correlation with osmolarity for EnvZ/OmpR ($R^2 = 0.066$) and CpxAR ($R^2 = 0.095$). For RcsCDB, there appears to be a correlation ($R^2 = 0.639$).

However, this is driven primarily by the strong response to the two highest osmolality conditions (NaCl and KCl), both of which are salts that produce a similar response. When these points are removed, there is no longer a correlation with the remaining chemicals. Thus, although each sensor is induced by high salt, we can conclude that none of the systems respond to osmolality.

Sensor Activity Patterns

Different patterns of sensor activity were produced in response to the various chemical stimuli. The time courses and patterns are shown in Figures 4 and S2. Of the seven possible non-trivial patterns of induction, five were observed. There are a few examples where chemically similar compounds produce different patterns. Notably, pentanol produces a pattern that is distinguishable from butanol (Fig. 4). This feature is a characteristic of olfactory systems and has also been observed for electronic noses (Doleman et al., 1998).

A PCA was applied to the data set in order to visualize the diversity and clustering of patterns (Fig. 5A). PCA reduces the dimensionality of data by finding new axes that capture the most information from the original data set (Jurs et al., 2000). For our data set, each pattern can be viewed as a vector with three elements. In this three-dimensional space, each chemical is represented by a point, and the position of the point along each axis corresponds to the induction of each of the three sensors. In this case, PCA reduces the dimensions from three to two and aids in visualization. Importantly, it could be applied to systems with many more sensors (e.g., all 32 *E. coli* sensors) in order to reduce the high-dimensional space. To capture differences in the patterns themselves, rather than differences in induction magnitudes, each pattern was normalized by its maximum before performing the PCA. As a result, a pattern made up of small inductions will cluster with a pattern of large inductions, as long as the ratio of activities between sensors are the same. Although PCA enables the visualization of clusters, the magnitudes of the components do not correspond to the magnitude of induction; note that the point corresponding to no induction for all three sensors is not (0,0) in PCA space. Rather, it is represented in Figure 5A by a black dot.

The chemicals were clustered in principal component space by the patterns they produce (ovals in Fig. 5A). The salts (NaCl and KCl) strongly induced RcsCDB in comparison to the other sensors. One cluster (pentanol, phenylethyl alcohol, pH10, acetone) represents a pattern where CpxAR is the most induced. The cluster containing indole and procaine induced EnvZ/OmpR and CpxAR. The short chain alcohols (methanol, ethanol, butanol) induced CpxAR, and RcsCDB. Only low pH induces EnvZ/OmpR and RcsCDB, but not CpxAR, an observation that has been made previously (Nakayama and Watanabe, 1995; Thomas and Booth, 1992). Some of the patterns that appear at the edge between clusters (notably acetone and pH 10) represent intermediates that are difficult to statistically distinguish between clusters.

Several chemically similar compounds do not appear in the same cluster. For example, most of the linear short chain alcohols occur together in a cluster, but pentanol and phenethyl alcohol produced different patterns. These alcohols could perturb the membrane differently; for example, it is known that the effect on membrane fluidity and lipid composition depends on alcohol chain length (Dombek and Ingram, 1984b; Ingram, 1976). Other clusters can also be rationalized based on the effect that the chemicals have on the membrane. For example, indole and procaine are in the same cluster, and both are believed to perturb the outer membrane (Garbe et al., 2000; Labedan, 1988). Some chemical clusters that are known to increase membrane fluidity, such as alcohols and acetone (Dombek and Ingram, 1984a).

Sensor Patterns as a Function of Chemical Concentration

The pattern of sensor activities could change as a function of the chemical concentration. In fact, this is a feature of combinatorial sensors, where chemicals are frequently perceived differently at different concentrations (Doleman et al., 1998; Hudon et al., 2000). To test this, the activity patterns were measured over a range of short-chain alcohol (ethanol, butanol, pentanol) concentrations and a range of pH values (Fig. S3). The cytometry distributions showed that the responses were graded as a function of concentration (Fig. S4). Each concentration produced an activity pattern that can be visualized in PCA space. This enables the visualization of how a pattern changes across a gradient of pH or chemical concentration. In Figure 5B, each of the four trajectories corresponds to one chemical and each point is a different concentration. A trajectory crossing the boundary of a cluster signifies that the pattern has changed. Interestingly, although the magnitude of induction for the sensors varied significantly, the patterns for the three alcohols remain constant. In other words, the ratio of the activities between sensors stays constant. In contrast, the pattern changes when pH is varied.

The trajectories of butanol and ethanol overlap across all concentrations. Neither trajectory crosses that of pentanol. Thus, the observation that pentanol is in a different cluster from the shorter chain alcohols is not an artifact of concentration. Regulatory interactions that are able to respond to ratios of sensor activity would be able to identify a pattern independently of its magnitude of induction, or in this case, the concentration of inducing chemical.

Discussion

The EnvZ/OmpR, CpxAR, and RcsBCD sensors all respond to many environmental stimuli and are involved in the regulation of diverse cellular processes. Here, we determined the extent to which they exhibit characteristics in common with combinatorial sensors. This work demonstrates that diverse patterns are generated in response to chemical perturbations and that each sensor provides unique information. The patterns are constant with respect to the chemical concentration and are not correlated with the osmolarity of the media.

The EnvZ/OmpR, CpxA/CpxR, and RcsC/RcsD/RcsB sensors are known to respond to physical properties of the membrane (Danese and Silhavy, 1998; DiGiuseppe and Silhavy, 2003; Nakayama and Watanabe, 1995; Majdalani and Gottesman, 2005). Temperature, pH, various chemicals, oxygen, and osmolarity all affect membrane properties, such as fluidity, permeability, potential, and lipid and composition, and protein composition (Dombek and Ingram, 1984b; Farr et al., 1988; Garbe et al., 2000; Kim et al., 2002; Sinensky, 1974; Zilberstein et al., 1984). Altered by a particular stimulus or micro-environment, the membrane may act as a sensor, whose combined set of properties is sensed by a set of two-component systems, and whose patterns are then used as inputs to various cellular programs.

The impact of alcohol chain length on membrane perturbation is well studied and is particularly interesting in the context of our observations. Longer chains influence fluidity further into the membrane than do shorter chain alcohols (Dombek and Ingram, 1984b). Also, the adaptive changes in lipid composition vary with chain length (Ingram, 1976). For alcohols with chain lengths 1–4, 18:1 fatty acid levels increase and 16:0 fatty acid levels decrease. For chain lengths 5–10 however, the opposite occurs with 18:1 fatty acid levels decreasing and 16:0 levels increasing. This correlates with our observation that pentanol clusters away from the shorter chain alcohols (Fig. 5A). The difference in patterns between pentanol and the lower chain alcohols is driven predominantly by a difference in the RcsCDB response. It is possible that RcsCDB is sensing only shallow changes in membrane fluidity, or else that it is sensitive to the ratios of these specific lipids.

To truly participate in combinatorial sensor, it would need to be shown that different patterns of sensor activity induce varied cellular responses. If each sensor is assumed to be digital (ON or OFF), then a set of N sensors produces 2^N patterns. In other words, for a system of three sensors, the cell would need to have a mechanism to distinguish between eight ON/OFF combinations of the sensors. Graded or biphasic responses increase the number of potential combinations. There are a variety of mechanisms by which a regulatory network could respond to a particular pattern of response regulator activities. The simplest arrangement is to have multiple operators within a promoter that bind to the different response regulators. For example, if a promoter is activated by response regulator A and repressed by B and C, this would allow the promoter to respond to an ON/OFF/OFF pattern. Different arrangements of the three regulators would allow other promoters to respond to different patterns. It has been shown that multiple transcription factors regulating a single promoter can lead to synthetic genetic logic gates (e.g., NOR, AND) based on the locations of the operators (Kalir and Alon, 2004; Setty et al., 2003).

There are many examples of promoters that are regulated in different combinations of the three response regulators in this study (Fig. 1B and Table S1) (De Wulf et al., 2002; Ebel and Trempey, 1999; Francez-Charlot et al., 2003; Shin and Park, 1995). Beyond the three systems in this work, most two-component systems participate in the co-regulation of promoters. Individual promoters are limited in the number of signals that can be integrated.

Combinatorial sensors may be favored by evolution because it allows a large number of chemicals, stresses, and environmental conditions to be detected by a limited set of sensors. This is in contrast to a system where each condition has its own specialized sensor. Another advantage of a combinatorial sensor is that it is simpler to evolve the ability to respond to new environmental conditions. Rather than creating a new sensor, regulation can be rewired to respond to a new pattern of activities. This could be as simple as modifying a promoter to add or remove response regulator binding sites (Perez and Groisman, 2009).

Combinatorial sensors have two advantages: (1) a small number of sensors can respond to a large number of stimuli, and (2) new stimuli can be sensed by responding to a new pattern of existing sensors, rather than building a new sensor. The disadvantage is that it is more difficult to respond to very low concentrations of a chemical. Thus, from the perspective of genetic engineering, combinatorial sensing is most appropriate for identifying a particular micro-environment, especially when it is poorly defined. Combinatorial sensing may also be a useful paradigm for the design of synthetic chemical sensors that have higher specificity. For example, in designing a protein sensor that binds butanol, it could be hard to achieve specificity from other alcohols. Additional specificity could be obtained by screening general sensors to identify a pattern of activity produced by a chemical and then applying genetic logic circuits to integrate that signal with the specific sensor. For example, to improve a butanol sensor, the *rprA* promoter and an AND gate could achieve higher specificity against pentanol. Thus, though the application of genetic logic, multiple general sensors can be integrated in order to achieve a higher degree of sensing specificity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The three two-component systems used in this study are shown. **A:** Each sensor responds to many stimuli and different combinations of the response regulators affect a variety of cellular processes. **B:** One mechanism to respond to a pattern is through the arrangement of multiple binding sites in a promoter. Several examples of promoters are shown where footprinting data is available and the response regulator either activates (green), represses (red) or has a biphasic (red/green) affect on expression. Details, including references used to generate these figures, are included in the Supplemental Information.

Figure 2.

The plasmid-based transcriptional reporters are shown. **A:** An example of the time course is shown for the pAC-Rcs reporter plasmid when uninduced (grey) and induced by 550 mM NaCl (black). The cytometry distributions are shown for induced and uninduced cells at the end of the time course. **B:** The dependence of each promoter on its cognate response regulator was determined by measuring its induction in a knockout strain. The controls were performed with a strong inducer of each system: (left) *pompC*, 3 mM indole; (center) *pcpxP*, 0.2% phenethyl alcohol; (right) *prprA*, 550 mM NaCl. Error bars are the standard deviation from the mean of two experiments performed on different days.

Figure 3.

The measurement of properties required for participation in a combinatorial sensor. **A:** Chemically-distinct compounds and shifts in pH induce all three systems, but to varying degrees. Each graph shows the fold-induction of the chemicals and pH changes in rank order for each sensor. **B:** The activities of the sensors are independent from each other. The correlation coefficients for each pair of sensors were calculated (pcpXP-pompC, $R^2 = 0.323$; prprA-pompC, $R^2 = 0.024$; prprA-pcpXP, $R^2 = 0.001$). The data is the average of two or experiments performed on different days, and error bars are the standard deviation from the mean. **C:** The sensor activities do not correlate with the osmolality of the media and chemicals.

Figure 4.

Time courses are shown for chemicals that are characteristic of the five observed patterns. Patterns are shown for 550 mM NaCl, 0.3% pentanol, 0.54% butanol, 3 mM indole, and pH 5 (the entire data set is shown in Fig. S2). The corresponding pattern is shown on the right, where the height of each bar is equal to the maximum induction measured over the 2-h time course. Points are the average of two or more experiments performed on different days, and error bars are the standard deviation from the mean. For the pH 5 condition, the response of *pcpxP* is less than one, which is demarcated by a star. The colors of the bars correspond to the clusters in Figure 5.

Figure 5.

A PCA is used to cluster the sensor activity patterns produced by each chemical. **A:** Each point corresponds to the pattern generated by a single chemical projected onto a two-dimensional principal component space. The points are clustered by the pattern produced (ovals). The colors of the clusters correspond with the patterns in Figure 4. The pattern corresponding to no induction for all three systems is represented on the graph as a black dot. Note that the value of the principal components is not a measure of the magnitude of induction. **B:** The effect of chemical concentration on the patterns is shown. A trajectory crossing the boundary of a cluster signifies that the pattern has changed. The patterns for the alcohols are insensitive to changes in concentration, whereas the pattern for pH changes dramatically. The concentrations of chemicals in the trajectories are (top to bottom): pentanol (0.16, 0.2, 0.24, 0.28, 0.32%), butanol (0.5, 0.6, 0.7, 0.8%), and ethanol (1.6, 2.0, 2.4, 2.8, 3.2%). The pH values are (top to bottom) 4, 5, 6, 7, 8, 9, and 10.