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The Role of Antibody Concentration and Avidity in Antiviral Protection

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Neutralizing antibodies are necessary and sufficient for protection against infection with vesicular stomatitis virus (VSV). The in vitro neutralization capacities and in vivo protective capacities of a panel of immunoglobulin G monoclonal antibodies to the glycoprotein of VSV were evaluated. In vitro, neutralizing activity correlated with avidity and with neutralization rate constant, a measure of on-rate. However, in vivo, protection was independent of immunoglobulin subclass, avidity, neutralization rate constant, and in vitro neutralizing activity; above a minimal avidity threshold, protection depended simply on a minimum serum concentration. These two biologically defined thresholds of antibody specificity offer hope for the development of adoptive therapy with neutralizing antibodies.

Antibody responses against chemically defined haptens, proteins, and pathogens have been well characterized, and the properties of polyclonal sera and monoclonal antibodies (mAbs) specific for these antigens have been studied in detail in vitro. Increased avidities and on-rates of antibodies have been postulated to provide increased in vivo effectiveness and protection (*1*). However, such a correlation has only rarely been analyzed for antibodies specific for, and protective against, infectious agents in vivo. Low-avidity (10^5 M^{-1}) opsonizing antibodies can protect against bacteria (*2*), and some studies have correlated in vitro virus neutralization titers with in vivo protection (*3*), whereas others have found no such relation (*4*). Avidity, on-rate, neutralizing activity, or antibody concentration have not previously been analyzed with respect to protective activity in vivo. We used a panel of mAbs (*5–7*) and polyclonal antibodies derived from high-titer secondary and hyperimmune responses to test whether characteristics of antibodies in vitro can predict protective efficiency in vivo—that is, whether increased avidity of immunoglobulin G (IgG) provides protection at lower serum concentrations.

VSV is a rhabdovirus closely related to rabies virus. It is highly neurotropic and may cause neurological disease and death in mice. Recovery of mice from primary infections or resistance against reinfection depends on neutralizing IgG antibody responses; $CD8^+$ T cells are not involved, whereas mice lacking B cells always die (*8, 9*). The surface envelope of VSV contains ~1200 identical glycoprotein molecules that form a regular and densely ordered pattern of spike tips; these tips are the only sites accessible to neutralizing antibodies (*10*). Neutralization of rhabdoviruses is mediated by the prevention of docking of the virus to cellular receptors. This requires a minimum of 200 to 500 IgG molecules bound per virion (*11*). The Fc portions of antibodies are not crucial for antiviral protection in vivo or in vitro (*8, 12*).

We previously described a set of virus-neutralizing mAbs derived from mice immunized with VSV serotype Indiana (VSV-IND) (*6, 7*). Virtually all of a collection of 62 mAbs that neutralize VSV bind to a single antigenic site on VSV-G comprising three overlapping subsites with avidities ranging from 2×10^7 M^{-1} to 9×10^9 M^{-1}

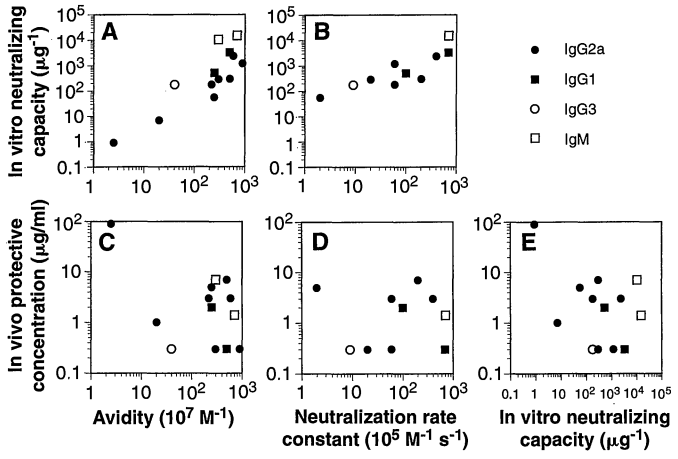

Fig. 1. Correlation of in vitro and in vivo parameters of mAbs. (**A** and **B**) Avidity (A) and neutralization rate constant (B) of mAbs, correlated with their in vitro neutralizing capacity. (**C** to **E**) Avidity (C), neutralization rate constant (D), and in vitro neutralizing capacity (E) of the same mAbs did not correlate with their in vivo protective concentration (see Table 1). Linear regression revealed correlation coefficients r of 0.86 (A), 0.93 (B), and <0.4 [(C) to (E)].

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(average avidity 2×10^9 M^{-1}). This panel of defined neutralizing antibodies was a useful tool to determine which of the known parameters—avidity, neutralization rate constant, specific in vitro neutralizing capacity, or serum concentration—was critical for antiviral protection in vivo. Avidities, neutralization rate constants [a direct measure of the on-rate (*13*)], and capacities to neutralize VSV in vitro are shown in Table 1 for the mAbs analyzed in this study. The in vitro neutralizing capacity correlated closely with avidity and with neutralization rate constant (Fig. 1, A and B). Intravenous VSV infection of SCID (severe combined immunodeficiency disease) mice, which lack B and T cells, reproducibly leads to a lethal encephalitis; this lethal infection can be prevented by passive immunization with intact neutralizing antibodies of various Ig classes and subclasses, or with antibody fragments (*9*, *12*).

SCID mice were treated with graded doses of mAbs. The measurable neutralizing antibody titer in serum revealed a calculated diffusion volume of ~2 to 3 ml for a mouse weighing 25 g (*9*). Five hours later, when a steady state of antibody concentration had been reached, mice were intravenously infected with 10^8 plaque-forming units (PFU) of VSV. Four days later, mice were killed and VSV titers in the brain were assessed. Surviving SCID mice that lacked detectable virus titers in the brain ($<10^3$ PFU per brain) were scored as protected. Representative experiments are shown in Fig. 2A. Using the 3-ml diffusion volume of mice, we determined the serum concentration needed for protection of 50% of the mice (Table 1). The minimal antibody concentration in serum necessary for in vivo protection was largely independent of subclass and of in vitro characteristics of the antibodies, namely, avidity, neutralization rate constant, and neutralizing capacity determined in balanced salt solution (Fig. 1, C and E). However, a minimal range of antibody concentration in serum of 0.3 to 7 μg/ml was needed for mAbs to be protective in vivo against 10^8 PFU of VSV-IND. As expected, protection was specific because a polyclonal serum against the New Jersey serotype of VSV (VSV-NJ) was not protective (Table 2).

One mAb (VI42) with an avidity of 2×10^7 M^{-1} was not able to protect mice against intravenous VSV infection, even at doses of up to 100 μg per mouse; this suggested that antibodies must have a minimal avidity of $>2 \times 10^7$ M^{-1} for effectiveness at the antibody doses tested. To analyze this avidity threshold further, we selected a VSV-IND escape mutant in vitro that was not neutralized by mAbs VI22 and VI41; this mutant was denoted VSV-TF (*14*). The panel of VSV-IND–specific antibodies was then tested for binding to this mutant virus by enzyme-linked immunosorbent assay (ELISA), and low-avidity antibodies were selected (Table 3). As in the first series of experiments, SCID mice were reconstituted with graded amounts of antibody and infected with VSV-IND or VSV-TF. None of the antibodies with avidities of $\leq 10^7$ M^{-1}, given at up to 100 μg per mouse, were protective after infection of mice with 10^8 PFU of VSV-TF. One mAb (VI32) with an avidity of 5×10^7 M^{-1} was protective against VSV-TF only at the very high dose of 100 μg/ml, confirming the range of the avidity threshold of $\sim 2 \times 10^7$ to 5×10^7 M^{-1} necessary for protection. Previous experiments documented in vivo protection by recombinant single-chain Fv antibody fragments and showed that complement and Fc receptors were not essential for mice to survive infection with VSV (*12*). Our findings add support to these results, because saturation of unoccupied Fc receptors of SCID mice by antibodies with normal mouse serum before the experiment did not change the protective capacity of the specific mAbs (*15*).

In normal mice, VSV does not replicate outside neurons. However, in mice lacking a functional interferon α/β system (IFNαβR$^{-/-}$), a low dose of VSV causes a generalized infection and virus replication in all tissues examined, leading to death within 2 to 3 days (*12*, *16*). Therefore, IFNαβR$^{-/-}$ mice offered a sensitive system

Table 1. Protective capacity of mAbs against VSV-IND. For details of the in vivo protection assay, see (*19*). The serum antibody concentration required for protection of 50% of passively immunized SCID mice (50% protective concentration) was identified by graphical extrapolation as illustrated in Fig. 2A, lower left panel, and division by the diffusion volume of a mouse (3 ml). For details of the in vitro analysis of antibodies, see (*13*). ND, could not be determined.

Clone	Subclass	In vitro			In vivo
		Avidity (M^{-1})	Neutralization rate constant (M^{-1} s^{-1})	Neutralizing capacity (μg^{-1})*	50% protective concentration (μg/ml)
VI22	IgG2a	9×10^9	6×10^6	1,200	0.3
VI42	IgG2a	2.5×10^7	ND	0.9	>30
VI41	IgG2a	2×10^8	ND	7	1
25G9	IgG2a	3×10^9	2×10^6	291	0.3
VI46	IgG2a	2.5×10^9	2×10^5	56	5
VI24	IgG2a	5×10^9	2×10^7	300	7
VI49	IgG2a	5.9×10^9	4×10^7	2,360	3
VI30	IgG2a	2.2×10^9	6×10^6	182	3
VI7	IgG1	5×10^9	7×10^7	3,300	0.3
VI29	IgG1	2.5×10^9	1×10^7	520	2
VI40	IgG3	4×10^8	9×10^5	177	0.3
G7G9E4	IgM	7×10^9	7×10^7	15,000	1.4
G14H3D7	IgM	3×10^9	ND	10,490	7

*Dilution of antibody with a starting concentration of 1 μg/ml yielding 50% plaque reduction in a standard neutralization assay.

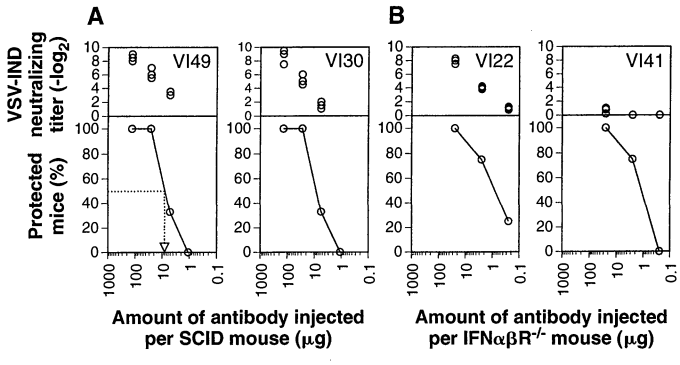

Fig. 2. Protective capacity of mAbs in mice. (**A**) SCID mice were reconstituted with graded doses of antibodies; 5 hours later, neutralizing titers were determined from 1:40 prediluted and 1:2 serially diluted sera (upper panel). Mice were intravenously infected with 10^8 PFU of VSV-IND, and 4 days later brains of surviving mice were assessed for the presence of virus (*19*). Mice without detectable virus were scored as protected (lower panel). One example of graphical extrapolation of the 50% protective antibody concentration is shown in the lower left panel (dotted arrow). (**B**) IFNαβR$^{-/-}$ mice were reconstituted with graded amounts of mAbs VI22 and VI41 (25, 2.5, or 0.25 μg), and neutralizing titers were determined from 1:40 prediluted and 1:2 serially diluted serum 5 hours later (upper panel). Mice were intravenously infected with 10^4 PFU of VSV-IND, and 3 days later the number of surviving mice was scored (lower panel) (*16*).

to assess in vivo protection against a low dose of VSV. IFNαβR$^{-/-}$ mice, which have B and T cells and normal Ig concentrations in serum, were reconstituted with graded amounts of mAbs VI22 (9×10^9 M^{-1}) and VI41 (2×10^8 M^{-1}), yielding either high or barely measurable neutralizing titers in sera (Fig. 2B). These mice were then intravenously infected with 10^4 PFU of VSV-IND, and survival was scored (Fig. 2B) (*16*). Although reconstitution with the low-avidity mAb VI41 led to a low neutralizing titer in serum (Fig. 2B), mice were protected, as were those that received the high-avidity mAb (VI22) and exhibited high neutralizing titers in serum (Fig. 2B).

An IgG concentration in serum of 0.3 to 7 μg/ml is equivalent to $\sim 10^{12}$ to 2.6×10^{13} molecules/ml, and 10^8 PFU of VSV-IND is equivalent to 1×10^9 to 3×10^9 virus particles displaying $\sim 1.3 \times 10^{12}$ to 4×10^{12} antigenic determinants, which indicates an almost equimolar ratio of antibody and antigenic determinants. It was therefore possible that in the protection assays, the effective antiviral antibody concentration in serum was reduced below the protective concentration immediately after the virus inoculum by absorption of the antibody by the virus. To exclude this possibility, we reconstituted mice with protective and subprotective concentrations of mAb VI10 (10 μg/ml and 1 μg/ml, respectively), infected them with VSV-IND, and determined virus neutralizing titers in serum before, 2 days after, and 4 days after infection. At a protective concentration of antibody, injection of 10^8 PFU of VSV-IND did not affect neutralizing titers in the sera of SCID mice; at subprotective concentrations of antibody, neutralizing titers decreased slightly during the course of the infection, by 1.8 dilution steps on average (Fig. 3). Similarly, neutralizing titers in the sera of IFNαβR$^{-/-}$ mice reconstituted with protective doses of antibody did not decrease upon injection of 10^4 PFU of VSV. Because of the generalized nature of the VSV infection, in IFNαβR$^{-/-}$ mice with subprotective concentrations of antibody, neutralizing titers in serum decreased by 3.5 dilution steps within 4 days (Fig. 3). However, no drop in titers could be observed when sera were analyzed 12 hours after infection. Thus, the lethal outcome of a VSV infection was not the result of extensive antibody consumption immediately after injection of VSV.

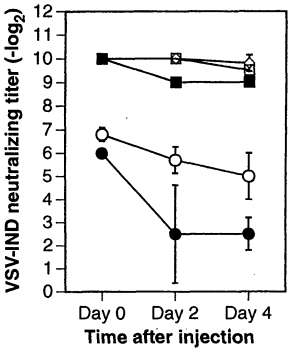

Fig. 3. Antibody consumption after VSV infection of passively immunized mice. SCID mice (open symbols) and IFNαβR$^{-/-}$ mice (solid symbols) were injected intraperitoneally with protective (squares, 30 μg per mouse) and subprotective doses (circles, 3 μg per mouse) of VSV neutralizing mAb VI10. SCID mice and IFNαβR$^{-/-}$ mice were infected with 10^8 and 10^4 PFU of VSV-IND, respectively. Mice were bled before, 2 days after, and 4 days after infection. Neutralizing titers were determined in a standard neutralization assay in serum. Titers of uninfected SCID mice exhibiting protective antibody concentrations are shown as controls (open diamonds).

Table 2. Protective capacity of polyclonal sera against VSV-IND and VSV-NJ.

Serum	Subclass	Average avidity (M^{-1})	Neutralizing titer	Protective amount of serum (μl)
Early d4 (IND)	IgM	5×10^9	1:40,000	10
Late d12 (IND)	IgG	3×10^9	1:100,000	0.4
Early d4 (NJ)	IgM	Low binding	No neutralization	>50
Late d12 (NJ)	IgG	Low binding	No neutralization	>50

These data confirm that the avidity and neutralization rate constant of antibodies correlate well with the neutralizing activity assessed in balanced salt solution in vitro (Fig. 1, A and B). However, no obvious correlation was found between minimal protective serum concentration in vivo and the in vitro parameters of avidity, neutralization rate constant, or neutralizing capacity (Fig. 1, C to E). This discrepancy cannot be attributed to IgG subclass differences because the eight IgG2a antibodies analyzed alone showed the same correlation (Fig. 1). Above a minimal avidity threshold of $\sim 2 \times 10^7$ to 5×10^7 M^{-1}, the antibody concentration (>1 to 10 μg/ml) seems to limit the protective effectiveness of antibodies in vivo. This avidity threshold corresponds to a dissociation constant of $\sim 2 \times 10^{-8}$ to 5×10^{-8} M, which is close to the measured protective IgG concentration of 0.3 to 7 μg/ml (2 μg/ml ≈ 10^{-8} M IgG). These IgG antibody concentrations must be reached within 5 to 6 days to protect mice against VSV (9). Antibodies below this avidity threshold seem to require very high in vivo concentrations for effectiveness (>30 μg/ml); such concentrations probably cannot be reached within the critical few days available for mice to survive. It is nevertheless interesting that the behavior of these low-avidity (but not of high-avidity) antibodies is apparently correctly described by the law of mass action, that is, low avidity can be compensated for by high concentrations. Collectively, our data reveal concrete threshold numbers for a protective immunity unit for antibodies (*17*).

It had been predicted that higher avidity of antibodies, with consequently lower concentrations necessary for protection, should improve antibody-dependent memory and might render it more economical (*1*). Our results suggest that maturation of avidity beyond the threshold of 10^7 to 10^8 M^{-1} may not improve protective capacity. Thus, there is a discrepancy between parameters defining the in vitro and in vivo activities of neutralizing antibodies. This discrepancy cannot be readily explained by uncertainties regarding the mechanism by which an-

Table 3. Protective capacity of mAbs against the virus variant VSV-TF. For details of the generation of VSV-TF, see (*14*). ND, not determined.

Clone	Avidity (M^{-1})		Amount injected (μg)	Animals protected	
	VSV-IND	VSV-TF		VSV-IND	VSV-TF
VI24	5×10^9	6×10^9	30	3/3	3/3
			3	2/3	3/3
VI10	3×10^9	3×10^9	100	ND	3/3
VI32	2×10^9	5×10^7	100	ND	3/3
			20	2/3	0/3
VI30	2×10^9	$<10^7$	70	ND	0/3
			20	2/3	0/3
VI22	9×10^9	$<10^7$	100	ND	0/3
			20	6/6	0/3
No antibody	–	–	–	0/6	0/6

tibodies neutralize VSV (*18*). It may be that the physicochemical properties of mouse serum and tissues in vivo are drastically different from the buffered saline conditions usually used in vitro. In particular, the kinetics of virus neutralization may be considerably slower in vivo than in vitro, because of complex diffusion kinetics of antibodies in blood and tissue lesions. Because the host-virus interaction is essentially a nonequilibrium system, these complex kinetics and their changes may drastically alter the net outcome of infection.

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5. The mAbs VI22, VI42, VI41, VI46, VI24, VI49, VI30, VI7, VI29, VI40, VI10, and VI32 (*6*) are representative for the steady-state day 12 and day 150 IgG response against VSV-IND. The mAb 25G9 [L. Lefrancois and D. S. Lyles, *Virology* **121**, 157 (1982)] was provided by L. Lefrancois. The IgM mAbs G7G9E4 and G14H3D7 were generated from mice infected with 2×10^6 PFU of VSV-IND 4 days previously.
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12. U. Kalinke *et al.*, *Eur. J. Immunol.* **26**, 2801 (1996). Although recombinant single-chain Fv antibody fragments protect against VSV infection if preincubated in excess with the virus, they are not able to protect in a passive immunization setting, because their half-life is too short.
13. Avidity, neutralizing capacity, and neutralization rate constant as a measure for the on-rate were determined as described (*6*). In brief, avidity was determined from unmanipulated hybridoma supernatants with a solid-phase ELISA, in which intact virus particles were coated. Within a factor of 2, the measured avidities of the antibodies were largely independent of incubation temperatures at 22° and 37°C. The avidities of some antibodies were validated with an in-solution competition assay by determining the concentration of viral glycoprotein in solution required for half-maximal competition of antibody binding in the solid-phase ELISA (*6*). Neutralization rate constants were determined using the kinetics of virus neutralization as read-out. These values reflect physicochemical on-rates. Because VSV is polyvalent, the neutralization rate constant might give slightly higher values than the on-rate [J. Foote and H. Eisen, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 1254 (1995)].
14. A VSV-IND variant was generated by growing 10^8 PFU of VSV-IND in the presence of mAb VI22 (150 μg/ml). The obtained virus was plaque-purified in the presence of mAb VI22 and subsequently grown in the presence of mAb VI41 (50 μg/ml). The obtained virus (VSV-TF) was then plaque-purified.
15. M. F. Bachmann, U. Kalinke, A. Althage, G. Freer, C. Burkhart, H.-P. Roost, M. Aguet, H. Hengartner, R. M. Zinkernagel, data not shown.
16. U. Steinhoff *et al.*, *J. Virol.* **69**, 2153 (1995); U. Müller *et al.*, *Science* **264**, 1918 (1994). Infection of $IFN\alpha\beta R^{-/-}$ mice with 10^4 PFU of VSV causes their death by day 2 to 3; protective concentrations of neutralizing antibodies extend survival to day 4 to 7. Because 1 PFU reaching nervous tissues will eventually be lethal, antibody concentrations about 5 to 10 times the protective concentration will usually prevent death after day 7.
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18. Neutralization requires covering of 30 to 50% of neutralizing sites (*11*). This is also illustrated by the observation that neutralization of VSV disappears within a 1:2 to 1:4 dilution step of the antibody, and in vivo protection vanishes in less than a 1:10 dilution step. This covering either blocks docking of VSV to receptors or inhibits fusion or pH-dependent conformational changes in the endosomes. All these mechanisms should operate similarly in vitro and in vivo. In this study, only the IgG2a-subclass antibodies analyzed confirm the conclusions drawn from all neutralizing IgG antibodies. Thus, the results seem not to be attributable to differences in specificity, nor to Fc and complement binding mechanisms, phagocytosis, or aggregations.
19. VSV-IND–specific neutralizing antibody titers were determined as described [M. F. Bachmann *et al.*, *J. Virol.* **67**, 3917 (1993)]. Viral titers in brains were determined as described (*9*). In brief, mice were exsanguinated under ether anesthesia and brains were removed aseptically. Brains were homogenized and viral titers were determined on Vero cells. To exclude the possibility that serum antibodies neutralized VSV particles in the brain after removal of the organ, we mixed VSV into the homogenized brain of a protected mouse and determined the viral titer. No reduction of VSV titer attributable to antibody in the brain was found. BALB/c (SCID) mice were obtained from the Institut für Zuchthygiene (Zürich, Switzerland) or from GSF GmbH (Oberschleissheim, Germany). Animal experiments were performed in accordance with Swiss federal law requiring use of minimal numbers of animals. VSV-IND (Mudd-Summers isolate) was originally obtained from D. Kolakofsky, University of Geneva.

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Bacterial Interference Caused by Autoinducing Peptide Variants

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The synthesis of virulence factors and other extracellular proteins by *Staphylococcus aureus* is globally controlled by the *agr* locus, which encodes a two-component signaling pathway whose activating ligand is an *agr*-encoded autoinducing peptide. The cognate peptides produced by some strains inhibit the expression of *agr* in other strains, and the amino acid sequences of peptide and receptor are markedly different between such strains, suggesting a hypervariability-generating mechanism. Cross-inhibition of gene expression represents a type of bacterial interference that could be correlated with the ability of one strain to exclude others from infection or colonization sites, or both.

"Bacterial interference" refers to the ability of one organism to interfere with the biological functioning of another. Although interference has been assumed to involve growth inhibition, this has been demonstrated in only a few instances (*1*, *2*). We now describe a type of bacterial interference in staphylococci that does not involve growth inhibition, but rather is mediated by inhibition of the synthesis of virulence factors and other extracellular proteins. Expression of the genes encoding these proteins is coordinately controlled by the *agr* locus (*3*–*5*) (Fig. 1), which consists of two divergent transcription units driven by promoters P2 and P3. The P3 transcript RNAIII, rather than any protein, is the effector of the *agr* response, which involves the up-regulation of genes encoding secreted proteins and down-regulation of genes encoding surface proteins (*5*, *6*). The P2 operon contains four genes—*agr*B, D, C, and A—all required for transcriptional activation of the two *agr* promoters (*4*). AgrC corresponds to the signal receptor and AgrA to the response regulator of a standard two-component signal transduction pathway (*4*). AgrB and D generate an autoinducing peptide that is secreted by the bacteria, can be isolated from culture supernatants, and is the activating ligand for AgrC (*7*). Addition of the autoinducing peptide to an early exponential phase culture of the producing strain causes the immediate activation of transcription from the two *agr* promoters (*7*).

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The existence of a form of bacterial interference involving this peptide was sug-