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A *Plasmodium falciparum* Antigen That Binds to Host Erythrocytes and Merozoites

Abstract. *Antigens that bind to erythrocytes were identified in the supernatant fluids of a cultured human malaria parasite* (Plasmodium falciparum). *A 175-kilodalton (175K) antigen bound only to erythrocytes susceptible to invasion. The 175K antigen from the Camp or the FCR-3 strain also bound to merozoites. However, the antigen did not bind to merozoites when merozoites and supernatant antigens were from different strains unless proteinase inhibitors were present. Moreover, erythrocytes coated with supernatant antigens from the Camp or FCR-3 strain were invaded normally by merozoites of the homologous strain but were partially resistant to invasion by merozoites of the heterologous strain. The 175K antigen may be a receptor acting as a "bridge" between erythrocytes and merozoites.*

DANIEL CAMUS*
TERENCE J. HADLEY
Department of Immunology, Walter Reed Army Institute of Research, Washington, D.C., 20307

*Present address: Faculté de Médecine, Lille and Unité INSERM 42, Villeneuve d'Ascq, France.

The human malaria parasite *Plasmodium falciparum* invades only human and a few nonhuman primate erythrocytes (*1*). The erythrocytes are invaded by the merozoite form of the parasite, and the process of invasion depends on the capability of merozoites to recognize ligands on the erythrocyte membrane (*2–4*). Identification of parasite receptors and their ligands is important for understanding the invasion process and for developing immunologic and chemotherapeutic weapons against the parasite.

Most studies of merozoite-erythrocyte interactions indicate that sialic acid and erythrocyte membrane glycophorins are important ligands for invasion (*3*). Using purified erythrocyte ligands as affinity substrates, investigators have tried to identify receptors on the parasite. Parasite antigens with molecular weights of 140K, 70K, and 35K were characterized by their binding to purified sialoglycoproteins of the erythrocyte membrane (*5*). However, the role of these antigens during invasion has not been determined. Parasite antigens of 155K and 130K bind to purified glycophorin A (*6*), but not to normal erythrocytes (*7*). Another 155K antigen is inserted into the erythrocyte membrane at the time of invasion (*8*, *9*), and specific antibodies purified from a human immune serum inhibit the invasion of erythrocytes by merozoites (*10*). However, these antibodies do not inhibit the attachment of merozoites to erythrocytes (*10*). Therefore, although the glycophorin-binding antigens and the antigen inserted into the membrane of ring-infected erythrocytes are probably involved in invasion, there is no evidence that they mediate the initial attachment of merozoites.

In this study we used intact erythrocytes as an affinity substrate to identify other parasite molecules that may be involved in initial attachment. This approach has the advantage of maintaining erythrocyte ligands in their natural environment and with their native configuration. Since the use of intact erythrocytes precludes the use of detergents, supernatant fluids of *P. falciparum* cultures were used as a source of antigens. This approach seemed feasible since many antigens, including the 155K antigens, are released into culture supernatants (*6*, *8*). Erythrocyte-binding antigens (EBA) were therefore investigated by incubation of culture supernatants with erythrocytes that were susceptible or resistant to invasion in order to determine the specificity of the binding.

Schizont-infected erythrocytes from synchronized cultures (*11*) were purified (*12*) and labeled with [^{3}H]isoleucine. Culture supernatants were collected after 4 hours. Erythrocytes were incubated with the labeled supernatants, washed, and lysed in Triton X-100. The EBA in this lysate were precipitated with serum from a Nigerian donor containing malarial antibodies and were visualized by fluorography after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (*13*).

Four antigens with apparent molecular weights of 175K, 120K, 65K, and 46K from culture supernatants bound to human and *Aotus trivirgatus* erythrocytes, both of which are susceptible to invasion (lanes b, e, i, and j in Fig. 1). Similar results were obtained with two different parasite strains, Camp and FCR-3 (*14*). No parasite antigens were detected in control experiments performed with serum from uninfected humans. The EBA were also investigated with erythrocytes treated with various enzymes and erythrocytes naturally resistant to invasion. No EBA were observed with human erythrocytes rendered resistant to invasion by treatment with trypsin (lane k in Fig. 1) or neuraminidase (lane 1 in Fig. 1). Binding of the 175K and 65K antigens did not occur with human Tn erythrocytes (lanes d and g in Fig. 1). These erythrocytes, which resist invasion, lack sialic acid on the *O*-linked tetrasaccharides of the glycophorins, but have sialic acid on *N*-linked oligosaccharides and on glycolipids (*15*). Erythrocyte-binding antigens of 120K, 85K, and 75K were revealed with guinea pig or rabbit erythrocytes, and EBA of 80K, 65K, and 46K were revealed with rhesus monkey

Table 1. Erythrocyte-binding antigens. Cells are defined as sensitive (S) or resistant (R) to invasion.

Type of erythrocyte	Sensitive or resistant	Molecular weight of EBA (K)	Lanes in Fig. 1	Remarks
Human, normal	S	175, 120, 65, 46	b, c, e, f	113K and 104K antigens bound to red blood cells of one of the seven donors (lanes c and f in Fig. 1)
Aotus trivirgatus	S	175, 120, 65, 46	i, j	Parasites cultured in human AB sera (*19*)
Human, chymotrypsin-treated*	S	175, 120, 46	m, n, o	Invasion reduced 29 percent at 1 mg/ml
Human, Tn†	R	120, 46	d, g	Lack sialic acid and galactose on glycophorin A; invasion reduced 90 percent
Human, trypsin-treated*	R	46	k	Invasion reduced 90 percent
Human, neuraminidase-treated*	R	46	l	Invasion reduced 95 percent
Guinea pig	R	120, 85, 75	p	Refractory to invasion
Rabbit	R	120, 85, 75	q	Refractory to invasion
Rhesus monkey	R	80, 65, 46	r	Refractory to invasion

*Erythrocytes (5×10^8 cells per milliliter) were incubated for 1 hour at 37°C with trypsin-TPCK (1 mg/ml, Sigma), neuraminidase (*Vibrio cholerae*, 50 unit/ml, Gibco), or chymotrypsin (0.05, 0.2, or 1 mg/ml, Sigma) in RPMI 1640 medium, washed, incubated with the appropriate protease inhibitor [soybean trypsin inhibitor (1 mg/ml) or 1 m*M* PMSF (Sigma)], and rewashed. †The percentage of Tn erythrocytes (95 percent) was determined by F. Klotz and G. H. Mitchell through an indirect immunofluorescence assay with a monoclonal antibody to immunoglobulin M (Gamma Biologics).

erythrocytes (lanes p, q, and r in Fig. 1). No 175K antigen bound to erythrocytes of guinea pig, rabbit, or rhesus monkey, which are naturally resistant to invasion. On the other hand, human erythrocytes treated with chymotrypsin at doses that do not inhibit merozoite invasion (0.05 and 0.2 mg/ml) were able to bind the 175K antigen (lanes m and n in Fig. 1).

We conclude that the binding of a 175K antigen to erythrocytes is correlated with their susceptibility to invasion (Table 1), suggesting that the antigen might be a parasite receptor. The lack of binding of the 175K antigen to neuraminidase-treated erythrocytes indicates that the binding site for this antigen on erythrocytes involves sialic acid. Moreover, the data obtained with human Tn erythrocytes suggest that binding of the 175K antigen specifically requires sialic acid on the *O*-linked tetrasaccharides of the erythrocyte glycophorins. The absence of binding of the 175K antigen to guinea pig, rabbit, and rhesus monkey erythrocytes indicates that the binding requires a conformation specific to human and *Aotus* erythrocytes.

Merozoites of the Camp strain were incubated with supernatants from cultures of parasites labeled with [^{3}H]isoleucine. A 175K antigen bound to the merozoites. The 175K merozoite-binding antigen was not observed when supernatants were absorbed on normal human erythrocytes, indicating that the erythrocyte-bound 175K antigen and the merozoite-bound 175K antigen were the same (lanes b and c in Fig. 2). Binding of the 175K antigen to merozoites cannot be explained by erythrocyte membranes contaminating the preparation, because infected erythrocytes were previously treated with trypsin (see legend to Fig. 2); however, binding to parasite structures contaminating the merozoite preparation cannot be excluded.

We next determined whether the 175K antigen obtained from one strain would bind to merozoites of the other. Camp or FCR-3 purified merozoites were incubated with radiolabeled supernatants from Camp or FCR-3 cultures. In the homologous systems (merozoites and superna-

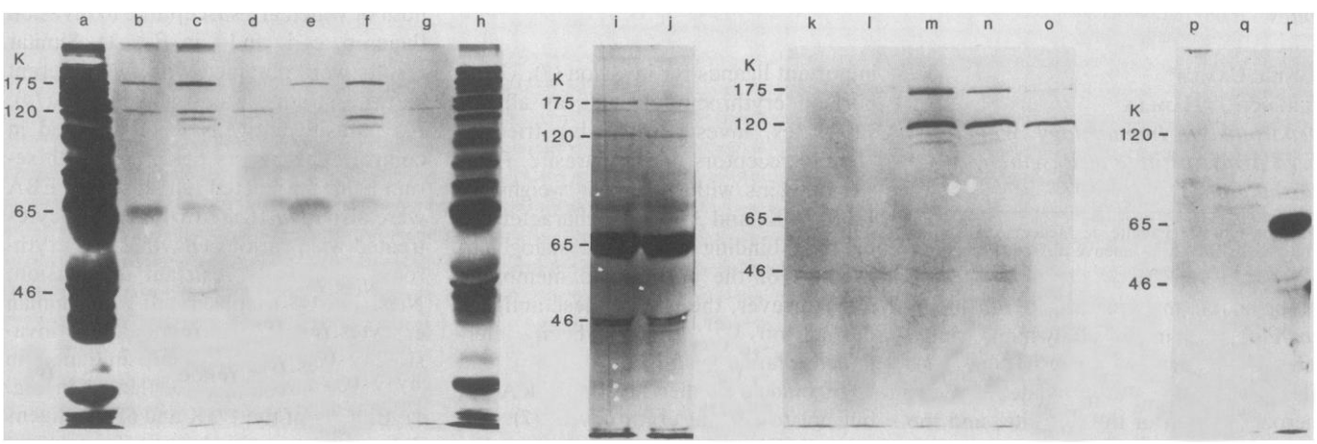

Fig. 1. Identification of EBA. Purified, schizont-infected erythrocytes (*12*) from synchronized cultures (*11*) were cultured (2×10^7 cells per milliliter) in medium containing 0.38 m*M* isoleucine and [^{3}H]isoleucine (100 μCi/ml; specific activity, 97 Ci/mmol; New England Nuclear). After 4 hours at 37°C, supernatants were collected by sequential centrifugations (400*g* for 10 minutes and 100,000*g* for 1 hour). Erythrocytes (2×10^9) were incubated with 1 ml of supernatant fluid (30 minutes at room temperature); washed three times in 15 ml of phosphate-buffered saline (PBS); treated with 200 μl of 2 percent Triton X-100 in 100 m*M* tris (*p*H 8.0), 1 m*M* EDTA, and 150 m*M* NaCl; and centrifuged (12,000*g* for 15 minutes). Human immune serum (75 μl) incubated with 375 μl of a mixture of protein A and Sepharose CL 4B (Pharmacia) was used to precipitate EBA from the erythrocyte lysate. Immunoprecipitated antigens were visualized by SDS-PAGE (*13*) and fluorography. Lanes a and h, antigens immunoprecipitated from supernatants of strains FCR-3 and Camp, respectively; lanes b and c, FCR-3 EBA, and lanes e and f, Camp EBA (normal human erythrocytes from two different donors); lane d, FCR-3 EBA, and lane g, Camp EBA (human Tn erythrocytes); lane i, Camp EBA, and lane j, FCR-3 EBA (*Aotus* monkey erythrocytes); lanes k to o, Camp EBA human erythrocytes treated with trypsin (1 mg/ml) (k), neuraminidase (50 unit/ml) (l), and chymotrypsin (0.05, 0.2, and 1 mg/ml) (m, n, and o); and lanes p, q, and r, Camp EBA (guinea pig, rabbit, and rhesus monkey erythrocytes, respectively).

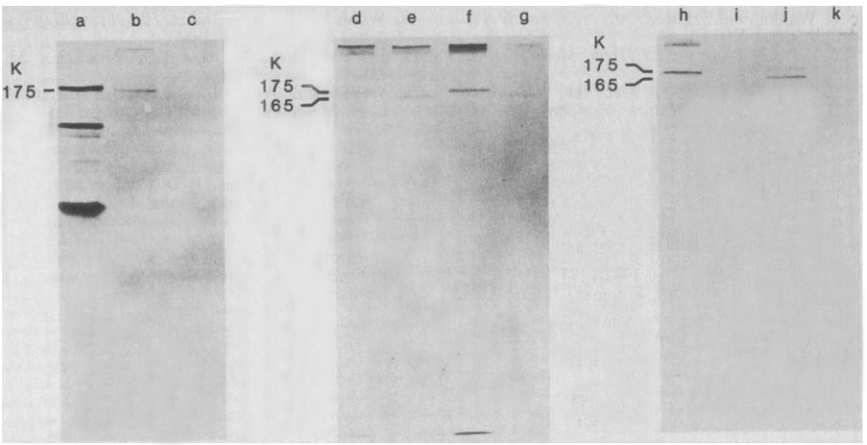

Fig. 2. Identification of merozoite-binding antigens. Schizont-infected erythrocytes (2×10^8) were purified from cultures treated with trypsin at the ring stage (*18*), incubated in 10 ml of culture medium for 2 hours, centrifuged at 400*g* for 10 minutes, and resuspended in 1 ml of culture medium for 1 hour at 37°C with rocking. Merozoites were separated from unruptured schizonts by a 5-second centrifugation in an Eppendorf model 5413 centrifuge, pelleted by an additional 15-second centrifugation, resuspended in radiolabeled supernatants (supplemented with 38 m*M* isoleucine) for 10 minutes at 37°C, washed three times in 15 ml of PBS, and extracted with 200 μl of Triton X-100. Merozoite-binding antigens were precipitated and analyzed as described for EBA. Lane a, control Camp EBA (human erythrocytes); lane b, Camp merozoite–binding antigens from Camp supernatants; lane c, Camp merozoite–binding antigens from Camp supernatants adsorbed on human erythrocytes for 1 hour at room temperature; lane d, Camp antigens binding to Camp merozoites; lane e, Camp antigens binding to FCR-3 merozoites; lane f, FCR-3 antigens binding to FCR-3 merozoites; and lane g, FCR-3 antigens binding to Camp merozoites. For lanes h to k, supernatants from strain Camp or FCR-3 were incubated with Camp merozoites in the presence of proteinase inhibitors [$2 \times 10^{-4}M$ L-1-tosylamide-2-phenylethyl-chloromethyl ketone (TPCK); $10^{-3}M$ 1,10-phenanthroline monohydrate; $2 \times 10^{-3}M$ phenylmethylsulfonylfluoride (PMSF); $5 \times 10^{-3}M$ EDTA; $2 \times 10^{-3}M$ *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK); and $10^{-3}M$ parachloromercuric benzoic acid (PCMB)—all from Sigma] introduced after 5 minutes of incubation. Lane h, Camp antigens binding to Camp merozoites; lane i, Camp antigens binding to Camp merozoites (supernatants adsorbed on human erythrocytes); lane j, FCR-3 antigens binding to Camp merozoites; and lane k, FCR-3 antigens binding to Camp merozoites (supernatants adsorbed on human erythrocytes).

tants of the same strain), merozoite-binding antigens of 175K were observed as before. In the heterologous systems, however, the 175K antigens were not observed, but antigens of 165K were (lanes d, e, f, and g in Fig. 2). A 175K antigen was observed in addition to the 165K antigen in each of the heterologous systems when proteinase inhibitors were introduced during incubation of supernatants with merozoites. Neither antigen was observed to bind to merozoites when the supernatants were absorbed with human erythrocytes (lanes h, i, j, and k in Fig. 2). Since no 165K antigen was observed to bind to erythrocytes and since absorption of supernatants on erythrocytes removed the 175- and 165K merozoite-binding antigens, it is likely that the 165K merozoite binding antigens are degradation products of the 175K antigens.

The effect of EBA on invasion was investigated with erythrocytes coated with supernatant antigens. Erythrocytes coated with antigens of each strain were invaded normally by parasites of the same strain, but were relatively resistant to invasion by parasites of the heterologous strain (Table 2). The decreased invasion rates observed in the heterologous systems were correlated with the absence of binding of intact 175K antigen to merozoites (unless proteinase inhibitors were present), suggesting that the 175K antigen is necessary for invasion.

We also investigated whether the 175K antigen is related to the 155K antigen inserted into the membrane of ring-infected erythrocytes (*8*, *9*) or to the 195K merozoite surface antigen (*16*). A rabbit antiserum against the 155K protein precipitated a 155K antigen from our supernatants but not any of the EBA. The 175K antigen and the 155K ring-associated antigen are therefore different. Similarly, four monoclonal antibodies against different epitopes of the 195K merozoite surface antigen failed to immunoprecipitate any of the EBA, although fragments of this protein (83K and lower) were found in supernatants (*17*).

On the basis of the specific binding of the 175K antigen to erythrocytes susceptible to invasion, its binding to merozoites, and the invasion assays, we suggest that these parasites release a 175K soluble receptor that mediates invasion by acting as a "bridge" between the erythrocytes and the merozoites. The 175K antigen may be involved in an initial step of recognition of the erythrocyte by the merozoite, while the 155K antigen may be involved in a subsequent step of invasion (*10*). An understanding of the molecules involved in invasion and their sequence of interactions may contribute to the development of a malaria vaccine.

Table 2. Invasion of EBA-coated erythrocytes. Human erythrocytes were incubated with supernatants from Camp or FCR-3 cultures for 1 hour at room temperature (5×10^7 erythrocytes per milliliter of unlabeled culture supernatant), washed three times in 15 ml of RPMI 1640 medium, and resuspended in culture medium for 1 hour at 37°C. Within each experiment, invasion assays were performed in triplicate wells of flat-bottomed microplates (Costar) with 10^7 erythrocytes (from a different batch for each experiment) and 5×10^5 schizonts per well in a volume of 200 μl. After a 16-hour incubation, during which ring forms appeared in newly invaded cells, 90 percent of the culture medium was replaced by medium without isoleucine and 1 μCi of [^{3}H]isoleucine was added to each well. After another 24-hour incubation, during which the parasites matured to schizonts, cells were collected and the incorporated radioactivity determined (*11*). Average ^{3}H counts in controls were 50 to 55,000 dis/min for Camp and 41 to 48,000 dis/min for FCR-3. Results were calculated as the ratio of the average radioactivity incorporated in cultures with treated erythrocytes to the average radioactivity incorporated in cultures with untreated erythrocytes. Control values are expressed as the mean percentage ± standard error of the mean; experimental values, as the mean percentage ± standard error of the ratio. Blood smears stained with Giemsa showed similar results.

Treatment of erythrocytes	Schizonts	
	Camp	FCR-3
	Experiment 1	
Control	100 ± 2.12	100 ± 2.73
Camp EBA	112 ± 4.89	59 ± 6.73
FCR-3 EBA	51 ± 2.02	107 ± 3.56
	Experiment 2	
Control	100 ± 3.05	100 ± 3.28
Camp EBA	112 ± 5.10	54 ± 5.11
FCR-3 EBA	56 ± 3.18	110 ± 4.37
	Experiment 3	
Control	100 ± 2.66	100 ± 4.18
Camp EBA	118 ± 5.52	61 ± 7.13
FCR-3 EBA	48 ± 3.41	103 ± 6.33

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A Common Origin of Rickettsiae and Certain Plant Pathogens

Abstract. *On the basis of ribosomal RNA sequence comparisons, the rickettsia* Rochalimaea quintana *has been found to be a member of subgroup 2 of the* α *subdivision of the so-called purple bacteria, which is one of about ten major eubacterial divisions. Within subgroup* α*-2,* R. quintana *is specifically related to the agrobacteria and rhizobacteria, organisms that also have close associations with eukaryotic cells. This genealogical grouping of the rickettsiae with certain plant pathogens and intracellular symbionts suggests a possible evolution of the rickettsiae from plant-associated bacteria.*

WILLIAM G. WEISBURG
CARL R. WOESE
Department of Genetics and Development, University of Illinois, Urbana 61801
MICHAEL E. DOBSON
EMILIO WEISS
Naval Medical Research Institute, Bethesda, Maryland 20814

The *Rickettsiaceae* (*1*) are small Gram-negative bacteria that, with few exceptions, are capable of growth only inside eukaryotic cells. They are associated with arthropods or other invertebrate hosts and often infect vertebrates. The group as taxonomically defined contains well-known human pathogens, such as the agent of epidemic typhus (*Rickettsia prowazekii*), Rocky Mountain spotted fever (*Rickettsia rickettsii*), and Q fever (*Coxiella burnetii*), as well as animal pathogens, such as agents of tropical canine pancytopenia (*Ehrlichia canis*) and tick-borne fever of sheep and cattle (*Ehrlichia phagocytophila*). A few species are epicellular in their natural environment and can be grown axenically (for example, *Wolbachia melophagi* and the genus *Rochalimaea*); others invade and grow in the cytoplasm of their host cells (genus *Rickettsia*); still others remain in the phagosome but do not stimulate a lysosomal response (genus *Ehrlichia*); and others are adapted to grow in the phagolysosome (*Coxiella burnetii*). The designation "rickettsia" or "rickettsia-like" is sometimes applied more broadly to include certain of the endosymbionts of protozoa, insects, and other invertebrates (*2*), as well as certain plant pathogens, such as the agent of Pierce's disease of grapevines (*3*). It is not known whether rickettsiae in this broadened definition thereof constitute a phylogenetically coherent (monophyletic) grouping. Nucleic acid hybridization studies (*4*) leave no doubt, however, that the genera *Rickettsia* and *Rochalimaea* are related to one another.

Table 1. Homology measurements for the sequences in Fig. 1. The lower left triangular section shows the percentage of total positions wherein any two sequences differ. Only those positions in the Fig. 1 alignment represented in all of the sequences are used in this analysis, a total of 1477. The upper right triangular section represents the number of positions (from the 1477) in which composition is both common and unique to a given pair of sequences.

Species	*R. quintana*	*A. tumefaciens*	*E. coli*	*B. subtilis*	*A. nidulans*
R. quintana	- - - -	94	6	10	17
A. tumefaciens	7.1	- - - -	8	4	11
E. coli	20.5	20.2	- - - -	27	46
B. subtilis	20.0	20.4	21.1	- - - -	63
A. nidulans	21.3	21.7	21.9	19.0	- - - -

Because of the difficulty associated with the culture of pathogens such as the rickettsiae, relatively little is known about their biochemistry and molecular biology. If free-living, readily culturable, close relatives of the rickettsiae can be found, many of their properties might be easily studied in these more readily manipulated systems.

Comparisons of nucleic acid sequences provide the best method for ascertaining microbial phylogenies. Partial sequencing of ribosomal RNA's (rRNA's) has, over the last decade, begun to reveal the evolutionary relationships among the bacteria (*5*). If one uses full sequencing methods (*6*), these relationships can be further refined and the deeper ones, such as the branching order among the various eubacterial divisions, can now be determined. A precise phylogenetic placement of *Rochalimaea quintana*, the subject of this report, demonstrates the power of sequencing approaches to (microbial) taxonomy.

Figure 1 shows the sequence of the *R. quintana* 16*S* rRNA gene aligned with four other eubacterial sequences, from *Agrobacterium tumefaciens* (*7*), *Escherichia coli* (*8*), *Bacillus subtilis* (*9*), and *Anacystis nidulans* (*10*). The percent difference between the various pairs of sequences is shown in the lower left triangular portion of Table 1. The numbers in the upper right triangular portion are tallies of the specific homology between them, that is, the number of positions in which composition is both common and unique to a given pair of sequences. (These numbers are basically a measure of derived characters, characters specific for particular lines of descent.)

The *R. quintana* and *A. tumefaciens* sequences (7 percent difference between the two) are much closer to one another than any of the other pairings in Table 1 (19 percent difference or more). This nearly threefold difference makes it obvious, without any formal analysis, that *R. quintana* and *A. tumefaciens* are specific relatives of one another. The count of "derived" characters (upper right portion of Table 1) shows this specific relationship as well.

Agrobacterium tumefaciens and *E. coli* are members of the purple bacteria, which is one of about ten major divisions of eubacteria (*5*). *Agrobacterium tumefaciens* represents the so-called α subdivision of the purple bacteria (*11*) and *E. coli* its γ subdivision (*12*). Thus, not only are the rickettsiae typical eubacteria but also they can be placed in a particular subdivision, α, of a particular eubacterial division, the purple bacteria.